

**Macrophage Mediated
Endothelial Injury and
Proliferation in Renal Transplant
Rejection.**

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Abstract

Macrophages ($M\phi$) have previously been implicated in both acute and chronic renal allograft rejection however the mechanisms remain unclear. In this thesis I set out to explore the effect of the $M\phi$ on the endothelium in the context of renal graft rejection.

Initial studies focussed upon human renal allograft tissue from transplant nephrectomies performed because of chronic allograft nephropathy (CAN). Immunostaining was carried out on these tissues (n=29) and control kidney tissue obtained from nephrectomies performed for renal cell carcinoma (n=19). An increased interstitial $M\phi$ infiltrate was found compared to control tissue. Immunostaining for the T cell marker CD3 and the B cell marker CD20 demonstrated that both lymphocyte populations were present in the CAN tissue with almost negligible numbers seen in control tissue. Previous work in the group had demonstrated a reduced number of CD31 positive peritubular capillaries in the tissues used in these studies. In the work undertaken in this thesis, additional analysis was performed to study lymphatic vessels. Immunostaining of control tissue with the lymphatic endothelial cell (LEC) marker podoplanin demonstrated a normal distribution of lymphatic vessels around large interlobular arteries. CAN tissue, however, exhibited an increased lymphatic density with lymphatic

vessels evident within the interstitium; a finding verified with two additional LEC markers (LYVE-1 and VEGFR-3).

Further investigations examined possible mediators that could be responsible for the reduced microvascular peritubular capillary network and increased lymphatic vessels present in tissues affected by CAN. Previous work had implicated nitric oxide (NO) generated by the enzyme inducible nitric oxide synthase (iNOS) in cardiac allograft rejection. Double immunolabelling for iNOS and the M ϕ marker CD68 revealed evidence of M ϕ expression of iNOS. No obvious reduction in vascular endothelial growth factor (VEGF)-A was evident although marked expression of VEGF-A was found in CD20 positive B cells within CAN tissue. Occasional interstitial cells expressed the lymphangiogenic growth factor VEGF-C, with double labelling studies indicating occasional CD68 +ve M ϕ that were positive for VEGF-C.

In vitro studies were undertaken to dissect the interaction between M ϕ and microvascular endothelial cells (MCEC-1) using well established *in vitro* co-culture techniques. Co-culture of cytokine activated bone marrow derived M ϕ with MCEC-1 cells (a murine cardiac microvascular endothelial cell line) resulted in increasing levels of MCEC-1 apoptosis and a reduced cell number over a 24-

hour time course. Non-activated Mø or cytokines alone were not cytotoxic. Co-cultures were performed in the presence of L-N-imino-ethyl lysine (L-Nil), a specific inhibitor of iNOS (control D-N6-(1-iminoethyl)-lysine (D-Nil)). L-Nil significantly inhibited MCEC-1 apoptosis and preserved cell number implicating a major role for NO in Mø-mediated MCEC-1 death. Importantly, L-Nil treatment did not affect TNF α production by cytokines suggesting that TNF α is not involved in MCEC-1 death in this *in vitro* experimental system.

Experiments were then undertaken involving the depletion of Mø in a murine model of acute renal allograft rejection. Renal transplants were performed between donor Balb/c mice and either FVB/N CD11b-DTR mice transgenic for the diphtheria toxin receptor (DTR) under the CD11b promoter or control non-transgenic FVB/N mice. Diphtheria toxin (DT) was administered on days 3 and 5 to induce Mø depletion and mice sacrificed at day 7. Isograft controls were also performed between FVB/N mice. Murine allografts exhibited marked interstitial F4/80 positive Mø infiltration with expression of iNOS in the allografts. There was significant loss of peritubular capillaries (PTC) in allografts compared to isografts, indicating microvascular injury. DT treated CD11b-DTR mice exhibited 75% reduction in Mø infiltration and this was associated with dramatic microvascular protection. B and T cells were not evident in the

isograft but significant accumulation of B and T cells was present in the allograft and not affected by Mø depletion. Interestingly, there was an increase in the number of podoplanin positive lymphatic vessels in the allograft compared to the isograft, which was significantly inhibited following Mø depletion.

The final area of study focussed upon attempts to isolate lymphatic endothelial cells *in vitro*. Two types of vascular cells (HUVECs and HDMECs) were analysed by flow cytometry for LEC markers and immunofluorescence to phenotype the cells. Magnetic bead sorting was then undertaken to isolate discrete populations of endothelial cells expressing LEC markers.

The murine studies reinforce the cytotoxic potential of Mø and supports a role for Mø in the deleterious rarefaction of microvascular interstitial vessels with resultant tissue hypoxia and ischaemia. Furthermore, these data support the involvement of Mø in the interstitial lymphangiogenesis that may occur in renal allografts. Furthermore, the study of human allograft tissue indicates that microvascular rarefaction and an increase in intrarenal lymphatic vessels occurs in human disease. Lastly, Mø expression of iNOS and VEGF-C suggests that Mø are involved in key processes that may adversely affect graft outcome.

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Declaration

I, Anya Adair, declare that this thesis was composed myself and the work contained herein is my own and was done by myself with the technical help of people I have acknowledged in the appropriate section, and this work has not been submitted for any other degree or professional qualification except as specified.

Anya Adair

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Abbreviations

%	: Percentage
°C	: degrees Celsius
A	: Absorbance
ABAM	: antibiotic antimycotic
APCs	: Antigen presenting cells
BMDM	: bone marrow derived M ϕ
BSA	: bovine serum albumin
BW	: body weight
cAMP	: cyclic adenosine monophosphate
CAN	: Chronic allograft nephropathy
CLEVER-1	: Combined lymphatic endothelial and vascular endothelial receptor
CNIs	: Calcineurin inhibitors
CO ₂	: carbon dioxide
CSF-1	: colony stimulating factor 1
DAB	: diaminobenzidine
DABCO	: 1-4-diazabicyclo-2-2-2-octane
DC	: dendritic cell
DMEM/F12	: dulbecco's modified eagle medium with F12
DMSO	: dimethylsulphoxide
D-NIL	: D-N6-(1-iminoethyl)-lysine
DT	: diphtheria toxin

ECGF	: Endothelial cell growth factor
ECM	: extracellular matrix
EDTA	: ethylenediaminetetraacetic acid
EFG	: epidermal growth factor
eNOS	: endothelial NOS
EtOH	: ethanol
ESRF	: End stage renal failure
FACS	: fluorescence-activated cell sorter
FasL	: fas ligand
FCS	: foetal calf serum
FITC	: fluorescein isothiocyanate
H ₂ O ₂	: hydrogen peroxide
HBSS	: hanks balanced salt solution
Hpf	: high power field
HUVECs	: Human umbilical vein endothelial cells
HDMECs	: Human dermal microvascular endothelial cells
I/R	: ischemia reperfusion
ICAM-1	: intercellular adhesion molecule 1
IFN- γ	: inteferon gamma
Ig	: immunoglobulin
IL	: interleukin
IMPDH	: Inosine monophosphate dehydroenase
iNOS	: inducible NOS

LEC	: Lymphatic endothelial cell
L-NIL	: L-N-imino-ethyl lysine
LPS	: lipopolysaccharide
	CD11b antigen (macrophage differentiation
MAC-1	: antigen)
LYVE-1	: Lymphatic Vessel Endothelial Receptor 1
MCP-1	: monocyte chemoattractant protein 1
MDCK	: madin darby canine kidney
M ϕ	: macrophage
MHC	: Major histocompatibility complex
Min	: minute
MMF	: mycophenolate mofetil
mTOR	: Mammalian target of rapamycin
NaCl	: sodium chloride
NK cells	: Natural killer cells
NFK β	: nuclear factor-kappa B
nNOS	: neuronal NOS
NO	: nitric oxide
NO ₂ ⁻	: nitrite
NOS	: NO synthase
PBS	: phosphate buffered saline
PBS -/-	: PBS without calcium and magnesium
PECAM-1	: platelet endothelial cell adhesion molecule 1

PGs	: prostaglandins
PMN	: neutrophil
QOL	: Quality of life
RT	: room temperature
TBS	: tris buffered saline
T _c	: cytotoxic T cell
TGF- β	: transforming growth factor beta
T _h	: T helper cells
TLRs	: Toll like receptors
TNF- α	: tumour necrosis factor alpha
TNFR	: tumour necrosis factor receptor
VSMCs	: Vascular smooth muscle cells
VCAM-1	: vascular cell adhesion molecule 1
VEC	: Vascular endothelial cell
VEGF	: Vascular endothelial cell growth factor
VEGFR	: Vascular endothelial cell growth factor receptor
vs	: Versus
VWf	: Von Willebrand factor
WT	: wild-type

Chapter 1.

Introduction

1.1 Kidney transplantation

1.1.1 Background

The first kidney transplant was performed between identical twins in 1954 (Tantravahi et al., 2006). Since then renal transplantation has become the treatment of choice for patients with end stage renal failure (ESRF) (Vathsala, 2005). It results in an increased life expectancy, and the overall risk of mortality is reduced by 80% at 12 months following transplantation when compared to patients who remain on dialysis (McDonald and Russ, 2002). This significant survival benefit also holds true in the elderly patient (Oniscu et al., 2004). In addition recipients experience a dramatic and all round improvement in quality of life (QOL) when compared to their dialysis dependent counterparts. They become more active and there is dramatic reduction in hospitalisation rates (Fiebiger et al., 2004; Nielens et al., 2001; Pietrabissa et al., 1992; Vathsala, 2005).

With the ever-increasing number of patients with ESRF and the limited supply of donor kidneys for transplantation it is essential that every renal transplant be optimised to achieve long-term patient and allograft survival. In recent years acute rejection rates have improved with the introduction of new immunosuppressants and one year survival is now routinely >90% (Cohen et al., 2006). Unfortunately this has not translated into an increase in allograft life span or a reduction in late graft loss rates (Afzali et al., 2005; Vathsala, 2005; Vuillemin et al., 1997). Besides death of a patient

with a functioning graft the most common cause of late graft loss is Chronic Allograft Nephropathy (CAN). I am aware that the terminology has recently changed but for the purpose of this thesis I will continue to use this term as this was used at the start of my studies. CAN accounts for 50-80% of graft failure in surviving recipients and results in an annual graft loss rate of 3-5% (Afzali et al., 2005; Joosten et al., 2005; Pascual et al., 2002). It is also the leading cause of graft loss in paediatric recipients (Alexander et al., 2006). CAN is the main cause of requiring return to dialysis and re listing following a transplant (Halloran et al., 1999). In this way it increases the burden on dialysis resources and adds to the ever-increasing transplant waiting list.

The two greatest dilemmas presently facing the renal transplant community are lack of organ availability and late graft loss. CAN is an irreversible process eventually resulting in return to dialysis and re transplantation and therefore poses a major problem in renal transplantation.

1.1.2 Acute Rejection

The Major Histocompatibility Complex (MHC) antigens (or Human Leukocyte Antigens (HLA) in humans distinguish self from non-self.

These MHC antigens expressed on donor cells act as targets to the recipient immune system and are responsible for initiating the rejection process. Acute rejection is primarily mediated through T cells. T cells recognise donor alloantigen in one of two ways and this stimulates the alloimmune response (fig 1-1).

Direct alloantigen recognition: Donor antigen is presented on donor antigen presenting cells (APCs) to recipient T cells in lymphoid tissue. This appears to be important in the early rejection process where many donor APCs have accompanied the graft into the recipient.

Indirect alloantigen recognition: Donor antigen is presented on recipient APCs to recipient T cells. This pathway may be important in maintaining the immune response. (Benichou et al., 1999; Martinez and Rosen, 2005; Tantravahi et al., 2006). The interaction between recipient T cells and donor antigens on APCs whether direct or indirect leads to T cell activation. T cell activation directly induces T cell proliferation and cytokine generation (importantly IL-2) leading to both humoral and cell mediated responses. This results in immune mediated allograft damage and ultimately acute rejection. (Fox and Harrison, 2000; Tantravahi et al., 2006).

The basis of immunosuppressive therapy is to inhibit activation of the immune system in response to donor MHC. Current immunosuppressive agents act by inhibiting T cell activation pathways either by disrupting IL-2 action or directly inhibiting cell proliferation.

Prednisolone disrupts cytokine pathways associated with T cell activation, particularly those induced through $\text{NF}\kappa\beta$. Calcineurin inhibitors (CNIs) have been pivotal in reducing acute rejection rates. The first CNI, cyclosporine was introduced into clinical practice in the 1980s. Tacrolimus followed and was first used in the transplant setting in 1990. They act by preventing transcription of the IL-2 gene. Unfortunately they carry significant nephrotoxic effects inducing afferent arteriolar vasoconstriction leading to reduced glomerular filtration rate and arteriolar hyalinosis leading to obliterative vasculopathy and tubulointerstitial damage (Tantravahi et al., 2006). Azathioprine and mycophenolate mofetil (MMF) prevent T and B cell proliferation by inhibiting purine biosynthesis (Allison and Eugui, 2005). Sirolimus prevents cell cycle progression by blocking the ability of the T cells to proliferate in response to IL-2. T cell populations can be reduced by using antibodies against T cell antigens e.g. thymoglobulin, monoclonal antibody OKT3 and monoclonal antibodies against IL-2 receptors (CD25) which inhibits T cell expansion. These antibodies may be used for induction therapy or to treat acute rejection (fig 1-1).

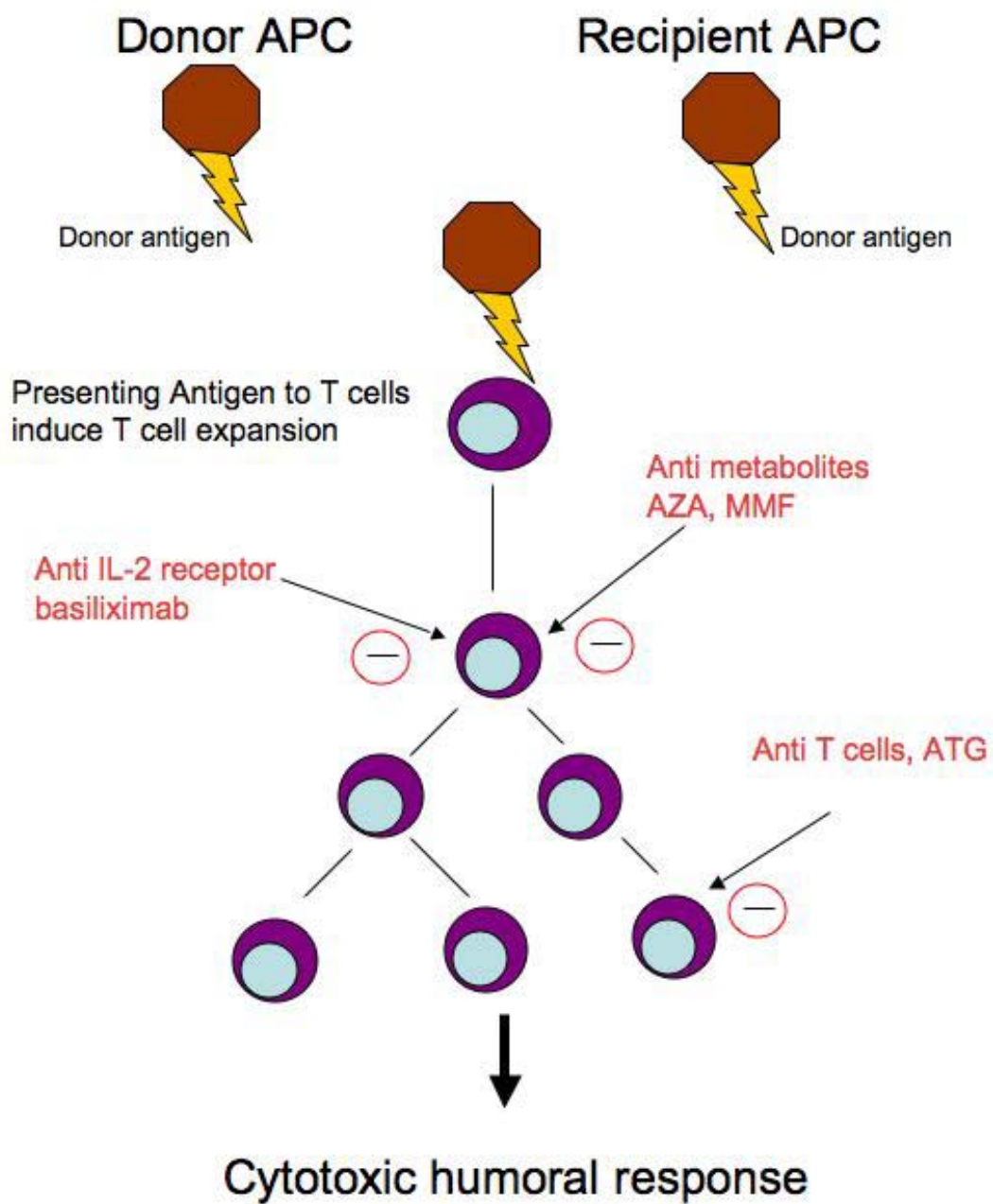


Figure 1- 1 Acute rejection is thought to occur via T cell activation and current immunosuppressant's target this pathway

1.1.3 Chronic allograft nephropathy (CAN)

CAN is the final common pathway of permanent renal allograft injury. It has characteristic histological features, which result from the accumulation of various insults to the transplanted kidney. These histological features are distinct from those of acute rejection, overt drug toxicity and recurrent or de novo specific disease entities (Joosten et al., 2003).

CAN is not synonymous with either chronic rejection which implies ongoing immunological activity or, chronic allograft dysfunction which suggests declining function, neither of which look at histological changes. (Nankivell and Chapman, 2006).

Histological Findings

The histopathological definition of CAN was developed from the Banff 1997 working classification of renal allograft pathology. Four international meetings have been held in Banff Canada since 1991 in an attempt to standardise renal transplant pathology interpretation and reporting (Racusen et al., 1999). The diagnosis of CAN is made on one or more of the following histological findings:

- Vascular changes - ranging from sub endothelial inflammation to intimal hyperplasia and ultimately atherosclerosis. These arterial wall changes are accompanied by infiltration of the vessel wall by macrophages (M ϕ) and foam cells (Elahi et al., 2006).

- Glomerular Lesions - including mesangial proliferation, thickening and duplication of the basement membrane and glomerulosclerosis associated with ischaemia
- Tubular atrophy - refers to the presence of tubules with thick redundant basement membranes, or a reduction of greater than 50% in tubular diameter compared to surrounding non-atrophic tubules.
- Interstitial Fibrosis - considered to be present when the supporting connective tissue in the renal parenchyma exceeds 5% of the cortical area.

(Joosten et al., 2003; Pascual et al., 2002; Yates and Nicholson, 2006).

The hallmark of CAN is an accumulation of extracellular matrix (ECM), which may occur in the interstitium, intima of the blood vessels, or in the glomerulus causing the characteristic histological changes described above. (Joosten et al., 2003; Pascual et al., 2002; Yates and Nicholson, 2006).

The severity of CAN is graded using the 1997 Banff Schema which

Takes into account the following histological lesions:

- Interstitial fibrosis
- Glomerulosclerosis
- Mesangial matrix increase
- Vascular fibrous intimal thickening
- Arteriolar hyaline thickening

The lesions are then scored according to a severity scale 0-3; absence of any of the prespective lesions – severe pathology. (Joosten et al., 2003; Pascual et al., 2002; Yates and Nicholson, 2006).

Clinical presentation

Clinically the typical course of CAN is a gradual decline in renal function occurring at least 3 months after transplantation, but may be as late as many years following transplantation (Joosten et al., 2005; Nankivell et al., 2003; Pascual et al., 2002). This loss of renal function is associated with falling creatinine clearance, a rise in serum creatinine and varying degrees of proteinuria and hypertension.

The diagnosis is often made late in the disease process as the clinical signs lag behind the histopathological changes. 76% of patients have evidence of CAN in protocol biopsies before clinical deterioration becomes apparent (Viklicky et al., 2003; Yehia et al., 2006)

Aetiological factors

The mechanism of acute rejection is considered to be predominantly an immune process. CAN however does not seem to have one single aetiology but instead is multifactorial with a range of responsible risk factors. These appear to be both alloantigen dependent and independent.

Alloantigen dependent:

- Acute rejection – late, multiple, and severe episodes are major risk factors. Acute vascular rejection carries a higher risk than cellular rejection.
- Poor HLA match
- Previous allosensitisation
- Inadequate immunosuppression.

Alloantigen independent /Non immune risk factors:

- Donor age - There is a direct correlation between poor long-term graft survival, the development of CAN and increasing age of the donor. This is attributed to the advanced age of the cells in general resulting in endothelial and epithelial atrophy and loss of function (Halloran et al., 1999; Yates and Nicholson, 2006).
- Ischaemia/reperfusion (I/R) injury - Endothelial cell injury leads to up regulation of adhesion molecules and infiltration and activation of leucocytes resulting in increased alloimmunity of the graft. This creates a proinflammatory and profibrotic state within the graft (Joosten et al., 2003; Pascual et al., 2002; Yates and Nicholson, 2006).
- Delayed graft function secondary to ischaemia reperfusion injury and acute tubular necrosis.

- Brain death and donor morbidity – brain death up regulates certain immune cell derived cytokines which increases solid organ immunogenicity (Yates and Nicholson, 2006).
- Calcineurin Inhibitors – have a deleterious effect on endothelial function. They also cause hypertension which is itself a risk factor for the development of CAN
- Recipient morbidity – hypertension in the recipient is significantly associated with CAN and late graft failure (Halloran et al., 1999)

The risk factors for the development of CAN occur at different time points throughout the life of the donor kidney (Nankivell and Chapman, 2006);

Pretransplant - Quality of donor tissue and the technical aspects of organ procurement and transplantation e.g. quality of organ perfusion.

Post transplantation

- Early e.g. acute rejection, delayed graft function
- Late e.g. subclinical inflammation/rejection (Moreso et al., 2006), hypertension, calcineurin inhibitor nephrotoxicity, Cytomegalovirus (CMV) (Halloran et al., 1999).

Nankivell et al put forward the theory that CAN consists of 2 distinct phases of injury:

- i. **Early** tubulointerstitial damage caused by I/R injury and immunological factors such as acute rejection.

- ii. **Later** micro vascular and glomerular damage and progressive interstitial fibrosis resulting from calcineurin toxicity and subclinical inflammation (Nankivell et al., 2003; Nankivell and Chapman, 2006).

1.1.4 Immunosuppressants and the treatment of CAN

The prevention of acute rejection has improved since the introduction of the calcineurin inhibitors (CNI). Currently, no immunosuppressive regimen has been effective in the treatment or prevention of CAN (Elahi et al., 2006). The options for treatment of this condition remain very limited (ACE inhibitors for the treatment of blood pressure are considered in those patients with hypertension and proteinuria), and experimental, with no general consensus between centres.

Calcineurin inhibitors (tacrolimus/cyclosporine) have been implicated as partly responsible for the fibrotic process seen in CAN (Afzali et al., 2005). Management of CAN has therefore concentrated on reducing exposure to CNIs by either reducing the dose delivered, with the risk of precipitating acute rejection or adding alternative immunosuppressants to enable either a reduction or complete withdrawal of CNI. Two options that are currently being investigated and used are a withdrawal of cyclosporine or tacrolimus and an introduction of either sirolimus or mycophenolate mofetil (MMF).

Sirolimus acts by binding to FK binding protein (FKBP). This complex then prevents progression of a cell from G1 to S phase of the cell cycle by inhibiting mTOR (mammalian target of Rapamycin).

In this way it acts both as an immunosuppressant and anti-proliferative agent with the ability of inhibiting fibrogenesis (Afzali et al., 2005). Trials have reported a significant improvement in renal function and creatinine levels (>50%) when patients are switched from CSA or tacrolimus (Diekmann et al., 2001; Dominguez et al., 2000). This may be due mainly to the withdrawal of CSA/tacrolimus rather than the introduction of sirolimus per se. The Leicester group carried out a trial of reducing CSA by 40% rather than complete withdrawal and failed to demonstrate any improvement in patient's histological state or renal function (sirolimus can enhance CSA nephrotoxicity) (Saunders et al., 2003). It is also important to remember that sirolimus has significant side effects including hyperlipidaemia, thrombocytopenia, skin rashes and deranged liver function tests.

MMF is a prodrug whose active metabolites inhibit the action of the enzyme inosine monophosphate dehydrogenase (IMPDH) required for the generation of purines in the S phase of the cell cycle. As with sirolimus it possesses both antiproliferative and immunosuppressant effects (Afzali et al., 2005).

MMF has been associated with improved late graft function when given in place of CsA (Meier-Kriesche et al., 2003; Ojo et al., 2000). Dudley

demonstrated that with the cessation of CsA and substitution with MMF, renal function stabilised or improved in patients who had biopsy proven CAN (Dudley et al., 2005). Again this improvement may be due more to the cessation of CsA rather than the action of MMF.

To date the trials into both these treatments are limited. In the main they have been conducted on a carefully selected group of patients and therefore are not truly representative of the true clinical setting. With sirolimus the studies have been small, mainly uncontrolled and with a short follow up. MMF studies are also small in number and again with short follow up, not extending beyond 2 years. With both these agents there is very little experience in patients with established CAN.

So CAN remains a significant challenge with persistent rates of graft loss despite new and highly effective treatment against acute rejection. For these reasons new strategies are required.

1.2 Macrophages

1.2.1 Background

Macrophages ($M\phi$) are present in low numbers within normal tissues throughout the body and act as 'sentinel cells'. These differ phenotypically and functionally from infiltrating Monocyte-derived $M\phi$ found in areas of local inflammation.

At the inflamed site activated endothelial, epithelial and mesenchymal cells release chemokines and cytokines that signal naïve monocytes e.g MCP-1 (Monocyte chemoattractant protein -1). Once monocytes leave the circulation and enter the tissues they differentiate into $M\phi$. Naïve $M\phi$ present at the inflamed site develop one of several phenotypic states dependent upon the nature of the microenvironment and activation stimuli.

This heterogeneous population of $M\phi$ are involved in the immune system. They contribute to the innate and acquired arms of the host immune response, the inflammatory response and tissue repair. They carry out a diverse number of roles including phagocytosis (Saville et al., 1989), antigen presentation and cytokine production with their response again being dictated by the mechanism of activation (Kluth et al., 2004; Wyburn et al., 2005).

Several states of $M\phi$ activation have been described including the following:

Classical activation was the first mechanism to be described. This requires $INF\gamma$ production by activated T cells and NK cells and the

presence of receptors able to recognise pathogen-associated molecular patterns e.g. TLRs (Kluth et al., 2004; Wyburn et al., 2005).

Classically activated M ϕ defend against invading microbes, activate the adaptive immune response by presenting antigen to helper T cells via IL-12 production and they produce cytotoxic agents e.g. nitric oxide, TNF α .

Alternative activation of M ϕ . These M ϕ are generated following exposure to IL-4 or IL-13 and do not release high levels of NO but increase expression of mannose receptor and MHC class II. They therefore have very limited cytotoxic ability, and are more involved with tissue repair and fibrosis (Jakubzick et al., 2003; Stein and Keshav, 1992; Stein et al., 1992).

Type II activation of M ϕ . This activation state is induced by stimuli such as LPS or CD40L in the presence of IgG immune complexes. These M ϕ are anti-inflammatory and release IL-10 but little IL-12 (Mosser, 2003).

M ϕ phenotype following apoptotic cell ingestion

M ϕ that phagocytose apoptotic cells also develop anti-inflammatory properties through their production of TGF- β , IL-10 and PGE₂. Conversely a proinflammatory response can be induced through the ingestion of necrotic cells (Golpon et al., 2004).

1.2.2 Macrophage infiltration correlates closely to cell apoptosis in renal disease

M ϕ have been found to play an important role in renal injury (Kluth et al., 2004). M ϕ infiltration correlates closely to vascular endothelial cell loss in various models of renal disease (remnant kidney model, unilateral ureteric obstruction, nephrotoxic tubular necrosis) (Kang et al., 2001a; Ohashi et al., 2000; Ohashi et al., 2002). In vitro studies indicate that cytokine activated M ϕ induce mesangial cell and epithelial cell apoptosis (Duffield et al., 2000; Kipari et al., 2006; Kipari and Hughes, 2002).

1.2.3 Macrophage infiltration associated with increased risk of acute and chronic allograft rejection

Traditionally, as previously stated it has been the T cell that has been implicated in the immune response and rejection process in transplantation. There is however evidence to support the involvement of the M ϕ in the process of organ rejection.

Experimental models:

In the rat cardiac allograft model apoptosis of the myocyte was paralleled by an increase in the number of M ϕ present (Szabolcs et al., 1996). In the rat model of renal allograft rejection Jose showed that administration of the M ϕ depleting agent clodronate ablated M ϕ and attenuated rejection and renal dysfunction (Jose et al., 2003).

Human studies:

Human studies looking at renal allografts have mirrored the above findings. Croker et al studied biopsies from patients with delayed graft function or rising plasma creatinine after a period of stable renal function. They showed that the M ϕ index (MI) provided a reliable indicator of graft survival with an increased MI correlating to reduced graft survival. (MI was taken to be the sum of M ϕ scores in glomerulitis, tubulitis, interstitial nephritis and endovasculitis) (Croker et al., 1996).

M ϕ have also been considered a marker of rejection with graft outcomes proving significantly worse if intraglomerular M ϕ are present (Ozdemir et al., 2002). In addition high M ϕ numbers have been noted in first biopsy specimens of patients going on to develop chronic rejection compared to those who continue to enjoy stable graft function (Pilmore et al., 2000; Srinivas et al., 2004).

1.3 Apoptosis

1.3.1 Background

Apoptosis occurs during development i.e. sculpturing tissues. In addition apoptosis can be detrimental e.g. tubular apoptosis in renal injury or beneficial e.g. PMN/ T cell apoptosis.

Apoptosis is a form of programmed cell death, which occurs during tissue remodelling, tissue injury and the resolution of inflammation. Apoptosis removes unwanted cells that are surplus to requirements or those that are potentially harmful e.g. T cells. (Hengartner, 2000; Savill and Fadok, 2000; Weitzman, 2004). Apoptosis is characterised by condensation and fragmentation of nuclear chromatin, blebbing of intact membranes and cell shrinkage and fragmentation (*Cailhier et al., 2006; Szabolcs et al., 1996*). These morphological changes that characterise apoptosis are brought about by a set of cysteine proteases, which belong to a protein family known as Caspases. (*Cailhier et al., 2006; Hengartner, 2000*).

Apoptotic cells are removed by the process of recognition and phagocytosis both by professional phagocytes i.e. M ϕ and non-professional neighbouring cells i.e. epithelial cells and fibroblasts (Platt et al., 1998). Apoptotic cell recognition depends on expression of cell surface signals e.g. the phospholipid phosphatidylserine on the apoptotic cell (Savill and Fadok, 2000). This then triggers engulfment of the cell (Weitzman, 2004). This phagocytic clearance

is essential in the process of resolution of the inflammatory response.

Phagocytosis of apoptotic cells prevents necrosis of the apoptotic cells which would lead to release of cytotoxic contents (Savill and Fadok, 2000; Weitzman, 2004) and suppresses the release of proinflammatory mediators such as $\text{TNF}\alpha$ by the proinflammatory $\text{M}\phi$ thereby modulating $\text{M}\phi$ phenotype (Savill and Fadok, 2000).

Apoptosis has previously been demonstrated in both normal developmental remodelling (Lang and Bishop, 1993) and various inflammatory states in the kidney (Duffield et al., 2000; Kipari et al., 2006). In allograft rejection Szabolcs showed apoptosis of cardiac myocytes and endothelium during cardiac allograft rejection (Szabolcs et al., 1996).

1.3.2 Mediators of Apoptosis

1.3.2.1 Nitric Oxide

Nitric oxide (NO) is generated from the conversion of L-arginine to L-citrulline, a reaction catalyzed by the enzyme nitric oxide synthase (NOS).

NOS isoforms: Three isoforms of NOS exist:

- A neuronal form (nNOS), producing NO, which is a neuromodulator.
- An endothelial form (eNOS) responsible for generating low levels of NO and maintaining low vascular tone.

- An inducible form (iNOS) first found and characterised in the M ϕ . It is induced by a number of cytokines and inflammatory stimuli including IFN γ , TNF α , LPS, IL-1 and plays a role in the inflammatory response (Guzik et al., 2003; Kluth et al., 2004; Nitsch et al., 1997; Romagnani et al., 1999). Nitric oxide is known to be produced by the classically activated M ϕ (Kluth et al., 2004).

Role of NO in experimental models:

In vitro iNOS generated NO has been shown to be a key death effector produced by the M ϕ during the induction of both renal tubular cell and mesangial cell apoptosis as well as during tubulointerstitial inflammation in vivo (Duffield et al., 2000; Kipari et al., 2006).

A substantial body of evidence points towards the involvement of NO in renal allograft rejection in various animal models, with high levels of interstitial iNOS expression in allograft but not isograft tissue. This expression has been localised particularly to infiltrating inflammatory cells and mononuclear cells. (Cattell et al., 1994; Worrall et al., 1995). Further evidence for a detrimental role of M ϕ produced NO comes from experiments showing a correlation between iNOS expression, M ϕ infiltration and the extent of apoptosis in the rat model of cardiac allograft rejection (Szabolcs et al., 1996). M ϕ depletion by clodronate and iNOS inhibition using aminoguanidine

attenuated the rejection process, though it should be noted that aminoguanidine is non-specific (Jose et al., 2003; Worrall et al., 1995).

The role of NO in human disease

The involvement of NO in the human setting has also been explored. Kashem showed that there was no interstitial iNOS expression evident in normal kidney (Kashem et al., 1996). However in glomerulonephritis iNOS was present particularly in areas of high M ϕ infiltration. In addition the renal biopsies of those patients with higher levels of iNOS expression exhibited greater renal damage and greater impairment of renal function (Kashem et al., 1996). In renal allograft rejection iNOS expression has been detected in the allograft and localised to infiltrating mononuclear cells (Romagnani et al., 1999). Also, increased levels of nitrite were noted in the urine of patients with rejection (Smith et al., 1996). Additionally high levels of serum nitrate and nitrite have been noted in patients undergoing acute rejection (Albrecht et al., 2000; Romagnani et al., 1999; Smith et al., 1996).

The role of NO in cell death or survival is concentration dependent. Physiologically low levels of NO inhibit T cell proliferation as well as cytokine and Fas-L induced apoptosis and is therefore cytoprotective. At high concentrations NO is cytotoxic. iNOS has

recently been found to be constitutively expressed in human renal tubules and glomeruli and Du et al demonstrated that iNOS expression in the donor kidney aids prolongation of graft survival in allogenic recipients (Du et al., 2006). Other studies as cited above suggest a destructive effect of iNOS generated NO on the allograft. Could this be explained by higher levels of NO being produced from M ϕ iNOS under circumstances with large M ϕ infiltrate? In addition this may be accompanied by a reduction in 'protective' renal tubular cell derived NO due to destruction of the tubular epithelium. In this way the balance between protective and cytotoxic NO production may be tipped in favour of the cytotoxicity in the rejecting allograft.

1.3.2.2 *TNF α and Fas-ligand*

Fas-L and TNF α are pro-apoptotic factors. Both have bioactive membrane bound forms, which induce apoptosis by binding to homologous receptors - Fas (CD95) and TNFR 1 and 2 respectively. Fas and TNFR1 initiate caspase dependent death signalling by similar mechanisms (Akyurek et al., 1998) (Boyle et al., 2003). Various previous studies have investigated the role of FAS-L and TNF α in cell apoptosis and graft rejection.

Animal models:

Akyurek et al showed in the rat aortic transplant model that Fas-L was mainly expressed by T cells and M ϕ . In this setting Fas mediated

apoptosis appeared to be involved in the lesions seen in chronic rejection (Akyurek et al., 1998). Soluble Fas blocks the binding of Fas to Fas-L and so acts as an apoptosis inhibitor. Wang et al blocked endothelial and smooth muscle cell apoptosis with over expression of soluble Fas and so reduced the development of transplant arteriosclerosis in the rat aortic transplant model (Wang et al., 2002).

In -vitro cell culture models

a) Murine

Gld/gld mice are homozygous for the point mutation in the Fas-L gene that results in a protein product unable to ligate Fas. Using bone marrow derived M ϕ (BMDM) from these *gld/gld* mice Duffield et al (Duffield et al., 2000) showed that NO independent apoptosis of mesangial cells was not mediated by M ϕ derived Fas-L. Co-culture with mesangial cells primed with TNF α suggested a role for TNF α in the induction of M ϕ mediated apoptosis in addition to NO.

b) Human

Using human M ϕ Boyle et al showed that M ϕ -derived TNF α contributed to M ϕ -induced apoptosis of vascular smooth muscle cells (VSMCs). This was achieved both through up regulating iNOS expression and NO production by M ϕ and increasing surface Fas-L expression. Blockade of the TNF receptors inhibited VSMC apoptosis (Boyle et al., 2003).

1.4 Lymphocytes

1.4.1 Background.

Lymphocytes are key cells of the adaptive immune system and impart specificity and memory to host defence as well as the capacity to discriminate between 'self' and 'non-self' from infectious pathogens providing self – non-self discrimination. After development from the myeloblastic stem cell those cells destined for the lymphocyte lineage undergo further maturation either in the thymus to form T cells or in the foetal liver and bone marrow to become B cells.

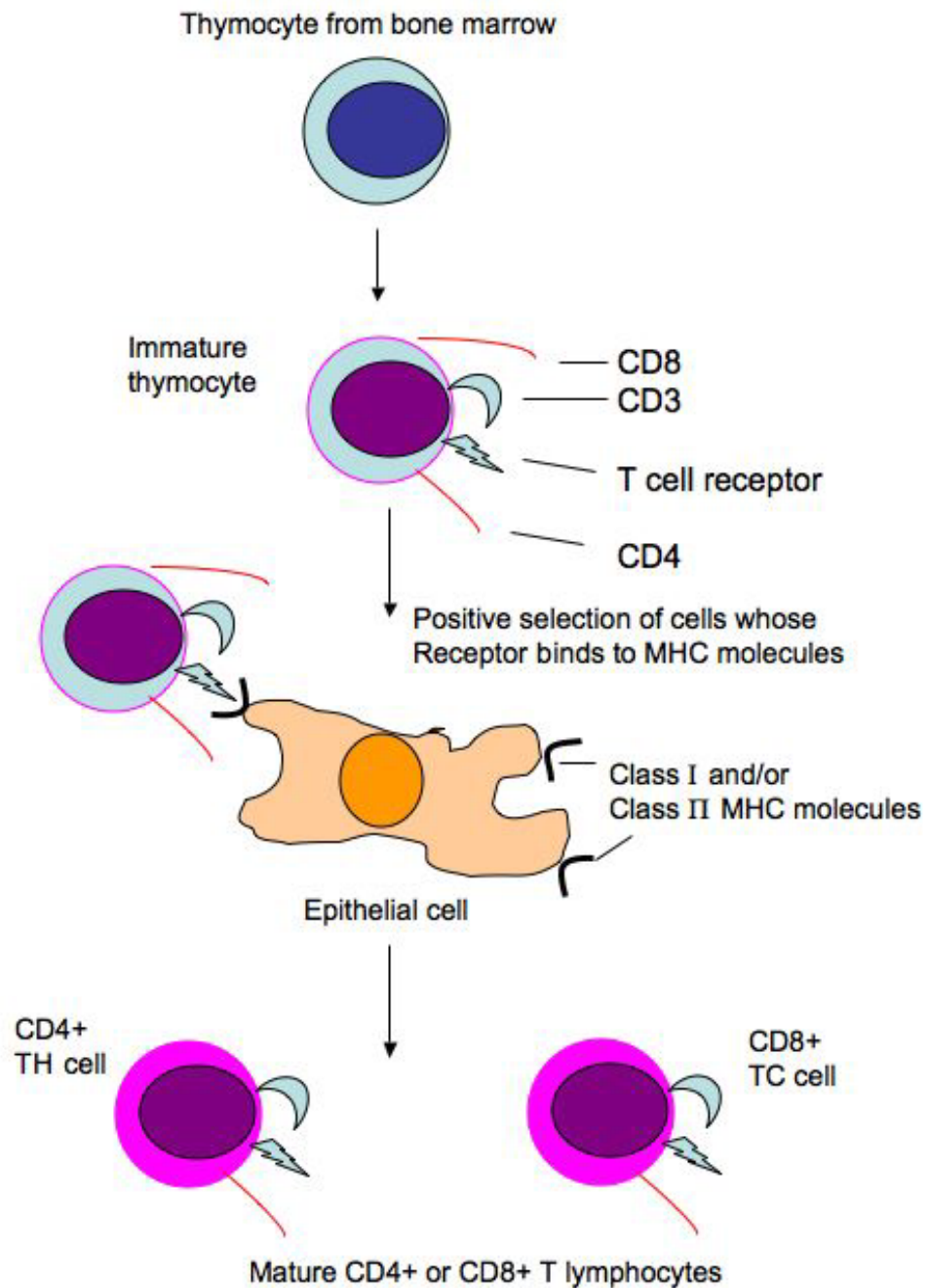
1.4.2 T cells

The maturation of T cells is characterised by a sequential appearance of certain cell surface molecules – CD3, T cell receptor (TcR), CD4 and CD8 (fig 1-2). TcR recognises peptide antigen that is presented by MHC molecules. TcR also require the physical contact with CD3, which is necessary for transmembrane signalling leading to T cell activation. The TcR is also complexed with one of two molecules from the immunoglobulin (Ig) superfamily (CD4 or CD8).

CD4 helper T cells interact with MHC class II found on specific antigen presenting cells – (B cells, M ϕ and dendritic cells). They are principally regulatory cells, which control the function of other lymphocytes. Two separate subclasses of CD4 helper T lymphocytes exist based on their differing cytokine profiles - Th1 cells produce

cytokines such as IFN γ resulting in cell mediated immunity involving generation of both specific cytotoxic T cells and activated M ϕ . Th2 cells on the other hand make cytokines such as IL-4, IL-5 and IL-6, which are critical for the induction of humoral immunity and B cell activation (Graca et al., 2002; Kupfer and Singer, 1989; Martinez and Rosen, 2005). CD8 cytotoxic or suppressor T cells interact with MHC class I expressed on most nucleated cells. These cells participate in cell-mediated immunity against infections, certain tumours and play a role in immune regulation (Kupfer and Singer, 1989; Moebius et al., 1991).

Figure 1- 2 T cell maturation (adapted from *Immunology* by Janis Kuby)



1.4.3 B cells

1.4.3.1 Antibodies and antigen presentation

B cells act both as antigen presenting cells (APCs) as well as differentiating into antibody producing plasma cells. Mature B cells express MHC class II molecules, CD20 and C3d. They can act as APCs by processing and presenting antigen complexed with MHC class II molecules. This complex is then presented to the cell surface to CD4 helper T cells. Consequently T cells are activated to produce cytokines such as IL-2, IL-4, IL-10 that further stimulate proliferation and differentiation of the B cells.

B cells intrinsically produce immunoglobins on their external membranes and once the mature B cell encounters the appropriate antigen the cell differentiates to form a plasma cell. These cells produce and secrete antibodies of one isotype with specificity for a single epitope. When antibodies bind to antigens they may neutralise pathogenic toxins, facilitate antigen ingestion by phagocytes (opsonisation), fix to and activate the complement system or participate in antibody mediated cellular cytotoxicity (Mosmann and Coffman, 1989).

1.4.3.2 *The B cell and lymphangiogenesis*

The inflammatory process is known to be associated with leukocyte inflammation. Previously work has focused mainly on the monocyte/M ϕ and T cells with little attention paid to the role of the B cell as an infiltrating cell. Recent studies looking at various models of renal disease including membranous nephropathy and renal allograft rejection have demonstrated the development of nodular infiltrates containing proliferating mature B cells expressing the chemokine receptor CXCR5 (Cohen et al., 2005; Heller et al., 2007; Kerjaschki et al., 2004). In addition, these nodular infiltrates were found to express the chemokine CXCL13 that is known to be associated with the accumulation of B cells expressing CXCR5 (Heller et al., 2007). This chemokine may therefore be important in the recruitment/accumulation of B cells within tissues.

Importantly these nodular infiltrates have been found to be in close proximity to areas of neo lymphangiogenesis, suggesting that B cells may be crucial in the formation of new lymphatics (Heller et al., 2007; Kerjaschki et al., 2004). This is in keeping with findings of previous studies, in which mice lacking CXCR5 are severely deficient in peripheral lymph nodes (Ansel et al., 2000).

Using a mouse model immunised with complete Freund's adjuvant (CFA), which induces an immune reaction in lymph nodes, Angeli et al observed that lymphangiogenesis and lymph node (LN) expansion occurred resulting in increased dendritic cell (DC) migration to

draining lymph nodes. This process appeared to be dependent on B cells entering the LN and promoting lymphangiogenesis. B cell dependent enlargement of LNs and lymphangiogenesis, which facilitates the mobilization of DCs to LNs, would allow the priming of a T cell response of increased magnitude.

Migration of APCs through lymphatics to draining lymph nodes represents a key step in the initiation of an adaptive immune response. Angeli et al provided evidence suggesting that the remodelling of the lymphatic network within the LNs enhances DC migration and that this process depends on the presence of B cells within the LN. In addition, VEGF-A expression was very prominently expressed by B cells within the lymph nodes supporting previous work indicating that VEGF-A may trigger lymphangiogenesis although the mechanism remains unclear (Hirakawa et al 2005: Nagy et al 2002: Ruddell et al 2003).

In addition to the above findings Angeli et al also found that blockade of VEGFR-3 (the specific receptor for VEGF-C & D but not VEGF-A) also diminished immunization induced lymphangiogenesis (Angeli et al., 2006). This is consistent with findings by Cursiefen et al in the mouse model of inflammatory neovascularisation of the cornea. This indicated that VEGF-A stimulates haemangiogenesis through direct activation of VEGFR-2 on blood vessels as well as lymphangiogenesis via activation of VEGFR-1 on M ϕ . These M ϕ proceed to produce VEGF-C leading to activation of VEGFR-3 on the

lymphatic vessel. This then induces lymphangiogenesis through the production of VEGF-C acting via its receptor VEGFR-3 (Cursiefen et al., 2004).

Selective blocking of VEGF-A using VEGF traps completely inhibited both haemangiogenesis and lymphangiogenesis and also reduced recruitment of inflammatory cells, specifically F4/80 positive M ϕ . In addition depletion of M ϕ either by γ irradiation or clodronate inhibited lymphangiogenesis (Cursiefen et al., 2004).

Considering all the above findings we can surmise that the B cells produce VEGF-A, which activates the M ϕ via VEGFR-1. Further speculation points towards these activated M ϕ going on to produce VEGF-C, which induces lymphangiogenesis via VEGFR3 (fig 1-3).

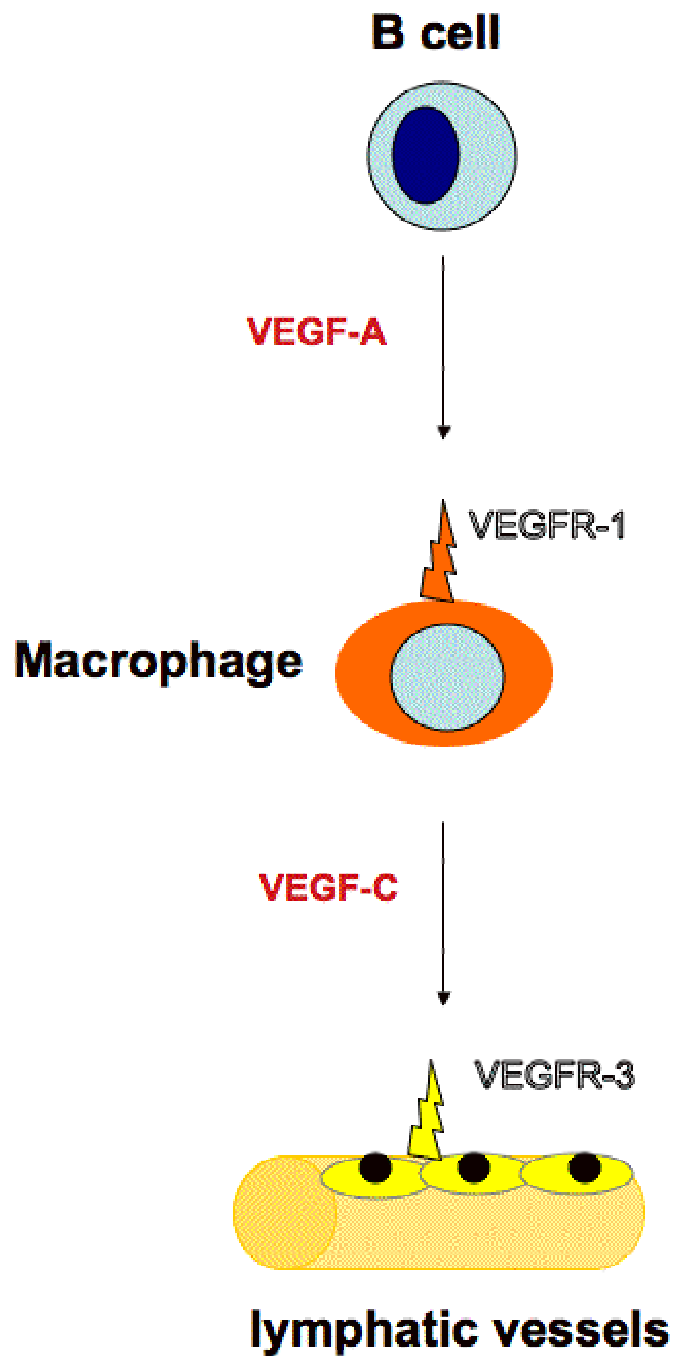


Figure 1- 3 The putative role of the B cell in lymphangiogenesis

1.5 The Endothelium

1.5.1 Background

Two distinct endothelial cell populations exist: vascular and lymphatic. These systems form non-anastomosing networks, which are functionally and structurally unique. Vascular endothelial lined blood vessels impart fluids, oxygen, nutrients, proteins and cells to the interstitium of the kidney whereas lymphatics serve as the exit route removing interstitial fluids and proteins. They also traffic APC to lymph nodes and are involved in tumour dissemination.

1.5.2 Vascular endothelial growth factor family and their receptors.

Vascular endothelial cell growth factor (VEGF) is a specific mitogen for endothelial cells. In mammals the VEGF family consists of 5 members; VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). These members' possess different physiological and biological properties and act through specific tyrosine kinase receptors; VEGFR-1, VEGFR-2 and VEGFR-3. VEGF-A, B and PlGF bind to VEGFR-1, VEGF-A also binds to VEGFR-2 and VEGF-C and D bind to both VEGFR-2 and VEGFR-3. This VEGF-VEGFR interaction is a key component in the process of angiogenesis and lymphangiogenesis (Olsson et al., 2006; Roy et al., 2006) (table 1-1).

Table 1- 1. Vascular endothelial growth factor receptors

Receptor	Expressed on	Ligand
VEGFR-1	Endothelial cells Pericytes Osteoblasts Monocytes and macrophages Renal mesangial cells	VEGF-A VEGF-B PlGF
VEGFR-2	Endothelial cells	VEGF-A VEGF-C VEGF-D
VEGFR-3	All endothelium during development. Lymphatic endothelial cells in adults	VEGF-C VEGF-D

VEGF-A was the first member of the VEGF family to be identified (Senger et al., 1983). It is a heparin binding growth factor and acts through specific tyrosine kinase receptors VEGFR-1 and VEGFR-2 (Roy et al., 2006). At least six different isoforms of VEGF-A are known to exist: VEGFA₁₂₁, VEGFA₁₄₅, VEGFA₁₆₅, VEGFA₁₈₃, VEGFA₁₈₉ and VEGFA₂₀₆ (Roy et al., 2006). The most common isoforms are VEGFA₁₂₁, VEGFA₁₆₅ and VEGFA₁₈₉ (Wakelin et al., 2004). VEGF-A is produced by a large variety of cells including M ϕ , myocytes, neutrophils, fibroblasts and epithelial cells (Pilmore et al., 1999). VEGF-A production is up-regulated by hypoxia, cytokines, NO and various growth factors (Pilmore et al., 1999) (Wakelin et al., 2004). It acts as an endothelial cell mitogen and angiogenic factor, modulator of vascular permeability and serves as a chemoattractant for both monocytes and endothelial cells (Ozdemir et al., 2005). The function of VEGF-A in health is not fully understood but it has been suggested that it maintains endothelial cell integrity (Pilmore et al., 1999). Several studies have found VEGF-A to be expressed in the normal human kidney, localising to the podocytes, distal tubular epithelium and collecting ducts (Ozdemir et al., 2005; Pilmore et al., 1999; Simon et al., 1995). However in pathological states it appears to be responsible for angiogenesis associated with both hypoxia and tumour growth (Folkman, 1995).

There are conflicting theories as to the production and action of VEGF-A during renal disease. The literature suggests that VEGF-A

expression by both infiltrating M ϕ and local tissues initially increases in an attempt to repair and protect, resulting in early angiogenesis in the development of human allograft rejection (Ozdemir et al., 2005; Pilmore et al., 1999). However renal fibrosis eventually develops (Ozdemir et al., 2002; Pilmore et al., 1999). Supporting this theory two studies demonstrated up regulation of VEGF-A in CAN tissue (Ozdemir et al., 2002; Pilmore et al., 1999). There was increased expression in the tubular cells in addition to expression in the glomeruli, mesangial cells, interstitium and vasculature. In addition the interstitial M ϕ appeared to be expressing VEGF-A. This is consistent with previous reports that VEGF-A expression is increased in chronic renal disease of various aetiologies and is associated with inflammatory M ϕ infiltration (Simon et al., 1995). In the advanced stages of disease there is an overall reduction in VEGF- A production by the local tissues potentially due to inhibition by M ϕ produced cytokines i.e. IL-1 β , IL-6, TNF α (Kang et al., 2002; Masuda et al., 2001; Ohashi et al., 2000; Ohashi et al., 2002).

VEGF-B: Two isoforms of this growth factor are expressed in humans: VEGFB₁₆₇ and VEGFB₁₈₆. VEGFB₁₆₇ accounts for over 80% of total VEGF-B and is expressed in most tissues including skeletal muscle, myocardium and brown fat. VEGFB₁₈₆ is found at much lower levels and only in a limited number of tissues. VEGF-B binds to the receptor VEGFR-1. Its precise role in vivo is not precisely known although suggestions include involvement in the formation of

coronary collaterals and inflammatory angiogenesis (Roy et al., 2006).

VEGF-C is a lymphangiogenic growth factor that acts via VEGFR-3 or -2 (Cursiefen et al., 2002; Roy et al., 2006). It is often associated with inflammation, wound healing and tumour metastasis (Angeli et al., 2006). VEGF-C promotes proliferation and survival of cultured human Lymphatic Endothelial Cells (LECs) (Makinen et al., 2001; Podgrabinska et al., 2002). This is in contrast to VEGF-A which acts as a survival factor and promotes the proliferation of both blood and lymphatic endothelial cells through VEGFR-1 and -2 (Podgrabinska et al., 2002). In the murine model of suture induced corneal inflammation lymphangiogenesis is also apparent in these vascularised corneas. Here VEGF-C was found to be expressed both by the lining of corneal lymph vessels as well as by infiltrating inflammatory cells. This VEGF-C expression was up regulated by pro inflammatory cytokines (Cursiefen et al., 2002). In the rat remnant kidney model interstitial mononuclear cells were found to express VEGF-C (Matsui et al., 2003). In a subset of acutely rejecting human renal allografts with nodular mononuclear cell infiltrates Kerjaschki found that a subpopulation of M ϕ within nodular infiltrates expressed VEGF-C (Kerjaschki et al., 2004).

Skobe et al demonstrated that when VEGF-C overexpressing melanomas were transplanted into nude mice there was

development of intratumoral lymphatics and enlargement of existing lymphatics at the tumour periphery (Skobe et al., 2001).

VEGF-D: This growth factor exhibits 48% homology to VEGF-C. It is expressed in many adult tissues including vascular endothelium, heart, skeletal muscle, lung and bowel (Roy et al., 2006). VEGF-D activates VEGFR-2 and VEGFR-3. VEGF-D is involved in endothelial cell proliferation and lymphangiogenesis (Roy et al., 2006).

PIGF: Placental growth factor was first identified in the placenta but has since also been identified in the heart and lungs, PIGF enhances angiogenesis through VEGFR-1 (Roy et al., 2006).

1.5.3 Identification and phenotyping of lymphatic endothelial cells (LECs) vs vascular endothelial cells (VECs)

Structurally the lymphatic capillaries are made up of a poorly developed basement membrane lacking pericytes. The lymphatic endothelial cells are connected directly to the interstitial collagen via anchoring filaments (Pepper and Skobe, 2003; Skobe et al., 2001).

Until recently, unlike the vascular endothelium, little was known about the lymphatic endothelium. This was due to lack of histological, ultrastructural and immunohistochemical (IHC) markers to differentiate between LEC and VEC (Al-Rawi et al., 2005).

Attempting to identify lymphatic vessels in routine histological sections relied on non-specific, suboptimal 'generalised' features. This included absence of erythrocytes in the lumen, a wide lumen

with a thin partly overlapping endothelial lining, absent tight junctions and an absent or discontinuous basement membrane. (Cursiefen et al., 2003).

Previously the only reliable modality to distinguish lymphatic from vascular structures was electron microscopy which showed the relative lack of basement membrane, fenestrations and cell junctions and their more prominent anchoring fibrils and vesicular transport system of LECs (Colvin, 2004). Although many endothelial cell markers exist and are used routinely in the identification and phenotyping of endothelial cells, they are non-specific and could not differentiate vascular from lymphatic endothelium. Recently specific (LEC) markers have become available and allow the separate identification and phenotyping of the two endothelial cell populations (table 1-2)

Table 1- 2 Vascular and lymphatic endothelial cell markers

Vascular endothelial cell marker	Lymphatic endothelial cell marker
<i>CD31</i> Transmembrane glycoprotein	<i>Podoplanin</i> Membrane glycoprotein
<i>CD34</i> Transmembrane glycoprotein	<i>Lyve-1</i> Hyaluronic Acid receptor protein
<i>Von willebrand Factor (VwF)</i> Glycoprotein	<i>VEGFR-3</i> Growth factor receptor
<i>Pal-E</i> Monoclonal antibody	<i>Prox-1</i> Transcription factor

CD31 is a member of the immunoglobulin super family and is a transmembrane glycoprotein. It is a constituent of the endothelial intercellular junction and plays a major role in the adhesion between the endothelial cell (EC) and recruited leukocyte during inflammation, and between ECs during angiogenesis. It is also present on the surface of platelets, monocytes, M ϕ and neutrophils (Pusztaszeri et al., 2006).

CD34 is a transmembrane glycoprotein and is present on ECs, leukaemic cells and stem cells. Although its function is still unclear, it is used in the diagnosis of leukaemia and the subclassification of vascular tumours (Pusztaszeri et al., 2006). It is used to purify progenitor cells.

Von Willebrand Factor (vWF) is a glycoprotein that mediates platelet adhesion to matrix e.g. collagen at sites of vascular injury and binds and stabilises factor VIII in the circulation. It is expressed on ECs as well as in the cytoplasm of megakaryocytes.

PAL-E is a monoclonal antibody that is specific for vascular endothelial cells of capillaries, medium sized and small veins and venules in frozen sections of human and some animal tissues. It does not stain the endothelial lining of lymphatic vessels. Using immunoelectronmicroscopy, the antigenic determinant recognized by PAL-E appears to be associated with endothelial vesicles.(Schlingemann et al., 1985).

Podoplanin: This is a glomerular podocyte mucoprotein involved in maintaining the glomerular barrier and the shape of the podocyte foot processes. It is also known to be expressed by lung alveolar type-1 cells, osteoblast and lymphatic endothelium (Al-Rawi et al., 2005; Breiteneder-Geleff et al., 1999). Schacht et al found that the D240 antibody specifically recognises human but not mouse podoplanin (Schacht et al., 2005).

This monoclonal antibody against sialoglycoprotein reacts with a fixation resistant epitope on the lymphatic endothelium found also on gonocytes of fetal testis (Arai et al., 2006; Fukunaga, 2005; Kahn et al., 2002). It has been found by several studies to be highly specific for lymphatic endothelium and unreactive to vascular endothelium (Arai et al., 2006; Galambos and Nodit, 2005; Kahn et al., 2002). As a result it has been used to separate mixed primary cultures of dermal endothelial cells into discrete vascular and lymphatic populations. (Kriehuber et al., 2001).

LYVE-1: Lymphatic vessel endothelial receptor-1 is expressed on the cell surface as a 60kDa protein. It is a major receptor for hyaluronic acid on the lymph vessel wall. It is a type 1 integral membrane polypeptide with 41% homology to the CD44 hyaluronic receptor (Al-Rawi et al., 2005; Banerji et al., 1999).

PROX-1: This transcription factor is a homologue of the Drosophila homeobox gene *prospero*. During early development prox-1 expression is induced on a sub population of endothelial cells

destined to become lymphatic endothelial cells. In the adult it is solely expressed by lymphatic endothelial cells (Al-Rawi et al., 2005; Cueni and Detmar, 2006).

VEGFR-3: Vascular endothelial growth factor receptor-3 is also known as Flt4. It is a member of the fms-like tyrosine kinase family and specifically binds to VEGF-C and VEGF-D but not VEGF-A. It is therefore a lymphatic specific growth factor receptor and in normal adult tissues its expression is predominantly limited to the lymphatic endothelium (Cueni and Detmar, 2006).

The introduction of these specific lymphatic endothelial cell markers has enabled the identification of distinct endothelial cell populations in tissues. (Kriehuber et al., 2001; Matsui et al., 2003). Also the isolation of these separate endothelial populations has become possible using antibodies to podoplanin (Kriehuber et al., 2001) Lyve-1 (Podgrabinska et al., 2002) and VEGFR-3 (Makinen et al., 2001) coupled with flow cytometric cell sorting or immunomagnetic cell separation methodologies.

1.5.4 Vascular endothelial cell proliferation and rarefaction

There is increasing evidence that the microvasculature plays an important role in progressive renal disease (Kang et al., 2001b) (Ohashi et al., 2002). Loss of capillaries would lead to reduced delivery of oxygen and nutrients to the tubules and interstitial cells, resulting in hypoxia and ischaemia of the tissues and eventual tissue fibrosis (Kang et al., 2001a).

The role of the vascular endothelium in the development of CAN is of particular interest as it forms the interface between donor and recipient cells and therefore is the first surface encountered by the recipient's immune system (Cailhier et al., 2006). Injury to the endothelium occurs as an early event, leading to the development of a hypoxic state with chronic ischaemia. (Elahi et al., 2006; Viklicky et al., 2003).

During renal disease it has been suggested that there may be an initial angiogenic response, possibly due to production of growth factors. Unfortunately this protective response is not sustained and is followed by progressive capillary loss (Ishii et al., 2005; Kang et al., 2001b; Ohashi et al., 2002). This is possibly due to loss of an angiogenic factor e.g. VEGF-A, or increased expression of an anti-angiogenic factor e.g. Thrombospondin-1 (TSP-1) (Kang et al., 2001a; Kang et al., 2001b).

Several studies have reported increased vascular density in renal disease (Ozdemir et al., 2002; Pillebout et al., 2001). Possible explanations for this initial increase in vascularity are:

1. Artifactual due to non specific endothelial cell markers which show a net increase in microvasculature due to an increase in lymphatic endothelial cells (Matsui et al., 2003).
2. Initially in response to hypoxia, reparative M ϕ are present from which M ϕ derived VEGF-A is expressed or which stimulate increased VEGF-A production by the local tissues.

This initial response is then followed by an increased number of pro-inflammatory M ϕ which inhibit VEGF-A production by the local tissues (Kang et al., 2001a) or have a direct cytotoxic effect on the VEC. In addition there is gradual destruction of key resident renal cells e.g. podocytes, which produce VEGF-A. All the above factors lead to an overall reduction in microvascular density.

1.5.5 Lymphangiogenesis

1.5.5.1 Background

The importance of the lymphatic system as a pathway for tumour metastasis has been well recognised. Many cancers initially spread through the lymphatics with the extent of lymph node involvement being important in tumour staging and assessment of prognosis. In this context lymphangiogenesis has already been reported (Pepper et al., 2003; Skobe et al., 2001). The mechanisms underlying the process of lymphangiogenesis and its importance in other diseases remain unknown.

1.5.5.2 Lymphangiogenesis and the M ϕ

Using various LEC markers, lymphangiogenesis has been found to occur in the peritumoral areas of several human tumours associated with tumour associated M ϕ (TAM) infiltration (Schoppmann et al., 2002). In addition lymphangiogenesis has been demonstrated in various inflammatory states (Pepper and Skobe, 2003; Skobe et al., 2001) and is associated with M ϕ infiltration and VEGF-C expression. Using the corneal suture model of inflammation, Cursiefen demonstrated increased lymphangiogenesis associated with VEGF-C expressing M ϕ . Clodronate mediated local depletion of M ϕ reduced corneal lymphangiogenesis (Cursiefen et al., 2002).

Using LEC markers it has been demonstrated that in normal kidney tissue the lymphatic vessels follow the larger intrarenal branches of

the renal artery to the level of the interlobular arteries (Kerjaschki et al., 2004). No lymphatics are normally found in the interstitium. However in states of inflammation there is an increase in lymphatic vessel density with lymphatics now being found within the tubulointerstitial space (Kerjaschki et al., 2004; Matsui et al., 2003). In both situations this was accompanied by M ϕ infiltration and VEGF-C expression.

1.5.5.3 *Where do these new lymphatics come from?*

One possibility already suggested is that M ϕ express VEGF-C which stimulates division of local pre-existent lymphatic endothelial cells acting through VEGFR-3 (Cursiefen et al., 2003; Kerjaschki et al., 2006; Kerjaschki et al., 2004; Makinen et al., 2001). Other theories are that a progenitor cell exists; possibly VEGFR-3 positive monocytes which transdifferentiate into new lymphatics (Kerjaschki et al., 2006; Maruyama et al., 2005). Kerjaschki et al studies on human gender mismatched renal transplants using in situ hybridisation for the Y chromosome suggest that these progenitor cells are recipient derived (Kerjaschki et al., 2006). An additional theory is that vascular endothelial cells transdifferentiate into lymphatics under the control of IL-3 (Grogger et al., 2004).

1.5.5.4 What is the role of these new lymphatic vessels?

We do not know the exact nature of the lymphatic drainage of the transplanted kidney. Previous auto- transplantation work on limbs in rats show that lymphatic flow is restored but may follow a more superficial route (Mobley and O'Dell, 1967). In addition, the lymphatics may play an active role in leukocyte transport. The glycoprotein **CLEVER-1** (common lymphatic endothelial and vascular endothelial Receptor-1), a member of the endothelial adhesion molecule family, is known to be expressed on lymphatic endothelium. CLEVER-1 mediates lymphocyte and peripheral blood mononuclear cell transmigration through the lymphatic endothelium and may play a role in the regulation of leukocyte egress from tissue (Salmi et al., 2004).

The Chemokine receptor CCR7 must be expressed by T lymphocytes in order to exit peripheral sites and to emigrate to draining lymph nodes. The CCR7 ligand CCL21 is known to be expressed by lymphatic endothelial cells in extra lymphoid tissues (Debes et al., 2005).

This suggests that transmigration/efflux of lymphocytes from tissues into the lymphatic system is a highly regulated process possibly under the control of the lymphatic endothelium itself. Thus the lymphatic endothelium may play an active rather than a passive role in this process.

Are these new lymphatics good or bad?

Lymphangiogenesis could feasibly be beneficial. New lymphatics may act to clear the inflammatory infiltrate from the kidney in an attempt to resolve the inflammatory process. Equally this extended lymphatic system could be harmful, with new lymphatics performing the following detrimental functions:

1. Allowing continuation of the inflammatory process.
2. Serving as pathways for the trafficking of antigen presenting cells thereby allowing these cells to migrate to lymph nodes and stimulate the adaptive immune response (Skobe et al., 2001).
3. Active recruitment of inflammatory cells by the lymphatic endothelial thereby setting up tertiary lymphatic organs in which the allogeneic response can continue (Kerjaschki et al., 2004).

1.6 Aims & Hypothesis

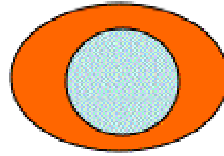
I set out to explore the involvement of the macrophage on the vascular and lymphatic endothelium in the context of renal allograft rejection.

The aim of this thesis was to investigate the following hypothesis:

- M ϕ induce vascular endothelial cell death by the production of pro-apoptotic death effectors such as NO, TNF α and FAS-L thereby contributing to microvascular rarefaction evident in transplanted organs.
- M ϕ support the production of new lymphatic vessels via the generation of specific lymphatic endothelial cell growth factors.

(fig 1-3)

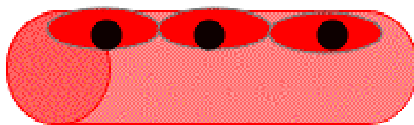
Infiltrating macrophage



Hypothesis

**M ϕ directly induce
Vascular endothelial
cell (VEC) death**

NO
TNF α
FasL

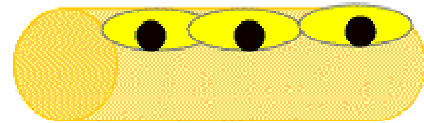


Promote vascular
endothelial cell apoptosis

Hypothesis

**M ϕ induce
Lymphatic endothelial
Cell (LEC) proliferation**

VEGF-C/D



Promote
lymphangiogenesis

Figure 1- 3 The hypothesis of this thesis.

Chapter 2.

Materials and Methods

2.1 Immunohistochemistry and immunofluorescence

2.1.1 Tissue specimens

Immunohistochemistry (IHC) and immunofluorescence (IF) were performed on 3µm tissue sections fixed in either 10% buffered formalin or methyl carnoy's solution (60% methanol, 30% chloroform and 10% acetic acid) and embedded in paraffin. Renal tissue from two species was studied:

2.1.1.1 Human:

Graft tissue available from 29 transplant nephrectomies diagnosed with CAN according to the Banff 1997 definition. Control tissue was obtained from the unaffected part of nephrectomy specimens from patients with a renal tumour (n=19) (see results section for patient details).

2.1.1.2 Murine:

2.1.1.2.1. Model of murine renal transplantation

Following administration of anaesthetic, donor kidney, ureter and bladder were harvested *en bloc* and transplanted onto recipient abdominal aorta, inferior vena cava and bladder. The right native kidney was removed at the time of transplantation whilst the left native kidney was left in situ. The experiment was terminated at day

7 when the histological picture was one of acute rejection (fig 2-1).

All surgical procedures were performed by Dr Qi.

Allograft: Donor kidneys from wild type male Balb-c mice were transplanted into a recipient male FVB strain. This provided an H2 mismatch and therefore at day 7 the histological picture was one of acute rejection (n=18).

Isograft: As non-rejecting isograft controls, kidneys were transplanted between male FVB litter mates and culled at day 7 (n=5) (fig 2-1).

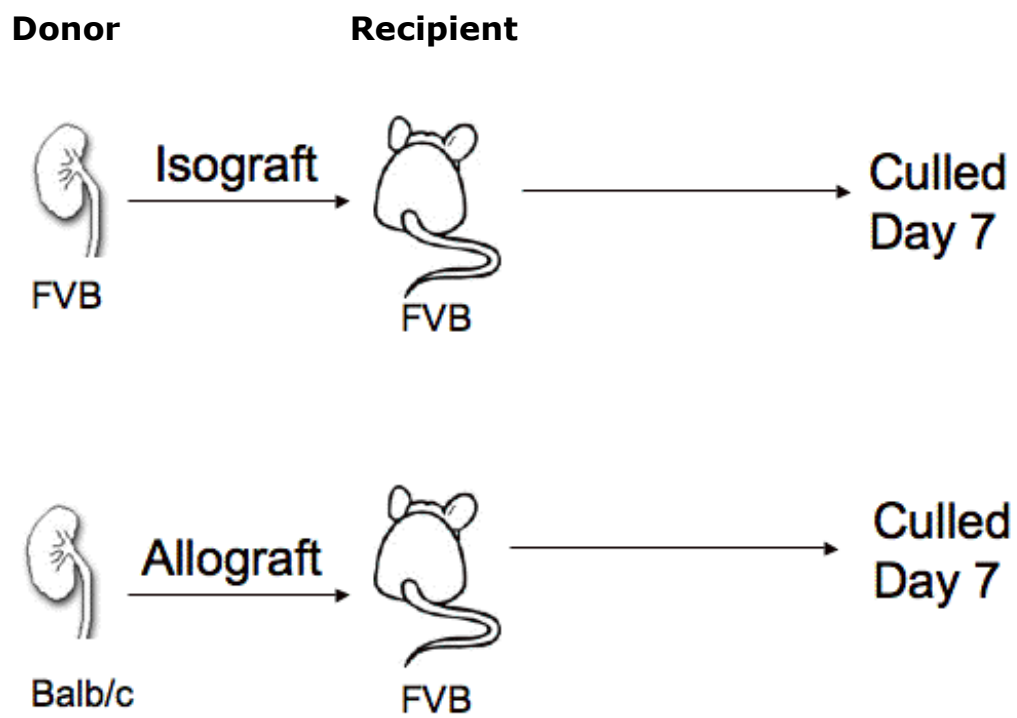


Figure 2- 1 Murine model of renal transplantation

2.1.1.2.2. *Conditional macrophage ablation in diphtheria toxin receptor transgenic mice.*

The expression of the human receptor for diphtheria toxin (DT) (also known as heparin binding epidermal growth factor (hbEGF)) in mouse cells confers sensitivity to DT *in vivo*. The human receptor is 1000 fold more potent compared to the murine receptor such that injection of DT will kill murine cells that express the human DT receptor. Conditional M ϕ ablation transgenic mice were generated using the construct CD11b-DTR. This used the CD11b promoter to provide M ϕ specific expression (Cailhier et al., 2005). The M ϕ and monocyte of this transgenic mouse (CD11b-DTR) specifically express the human diphtheria toxin receptor (DTR) such that monocyte/M ϕ can be specifically ablated by the intraperitoneal injection of diphtheria toxin (Cailhier et al., 2005; Duffield et al., 2005). In this way conditional M ϕ ablation is achieved following DT injection.

Donors were male Balb/c to recipient transgenic cd11b male DTR (n=10). Treatment dose of DT 20ng/g was administered on day 3 and 10ng/g on day 5 following transplantation. Our control allograft group was the transplantation of allograft donor male Balb/c to recipient FVB (n=18). DT was also given on day 3 and 5 to this control group in order to control for any non-specific effect of DT. The experiment was terminated at day 7 (fig 2-2).

Allograft: Donor – Balb/c male mice

Recipient – CD11b -DTR male mice (n=10)

or non transgenic FVB/N male mice (n= 18)

Isograft: Donor FVB/N male mice

Recipient FVB/N male mice (n=5)

2.1.1.2.3. *Specimens obtained from the murine transplant recipient.*

Renal tissue, blood and urine samples were collected from the murine recipient to be used for experiments as outlined below.

- Renal graft tissue was obtained at day 7 and used for IHC
- Blood was taken from the recipient for flow cytometric analysis of circulating M ϕ , T & B cells at day 7.
- Urine was collected from the recipient via a bladder stab for analysis of nitrite and creatinine concentrations at day 0 pre transplant and day 7 post transplant.

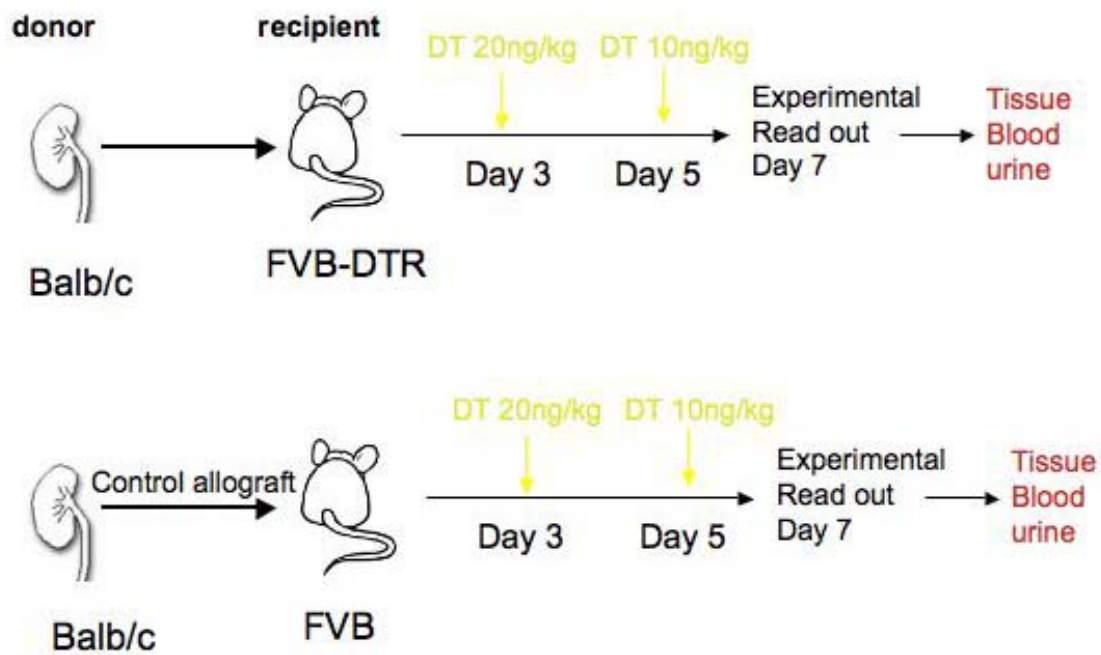


Figure 2- 2 Murine transplants using CD11b-DTR mice transgenic for the Diphtheria toxin receptor (DTR) as the recipient

2.1.2 Immunohistochemistry

2.1.2.1 Method for Immunohistochemistry (IHC)

Sections were dewaxed in xylene for 10 minutes and then rehydrated in descending concentrations of alcohol from 100% to 65% for 2 minutes each. Sections were then washed in deionised water prior to the appropriate antigen retrieval technique. This was microwave heat treatment at 1000 watts in 500mls of deionised water with 5mls of Vector Antigen solution (Vector Labs, UK) unless otherwise stated. Endogenous hydrogen peroxide activity was blocked by incubating sections in 2% hydrogen peroxide (Sigma, UK) for 15mins on a rocking platform. Sections were then loaded into a sequenza in Shandon cover plates (thermoelectron corporation, UK) and washed with PBS (Oxoid BR). Avidin block (Vector, UK) was applied for 10 minutes followed by a PBS wash and Biotin block (Vector, UK) for 10 minutes. Following a further wash in PBS, protein block (DAKO Cytomation, Denmark) was applied for 10 minutes before addition of the primary antibody diluted in antibody diluent (DAKO Cytomation, Denmark) (see table 2-1). After the appropriate incubation, the primary antibody was washed off and followed by application of the secondary antibody for 30 minutes at room temperature. Vector RTU peroxidase (Vector RTU Vectastain Kit, USA) was then applied for 30 minutes following a PBS wash. The substrate was then added – diaminobenzidine colour reagent (DAB) (DAKO Cytomation) for 5 minutes was used unless otherwise stated.

Counterstain was applied using haematoxylin and the slides were then cleared in increasing concentrations of alcohol (64%– 100%) and then into xylene. The slides were finally mounted on cover slips with mounting medium.

For methcarn sections the antigen retrieval step is omitted. Methcarn sections were used for all the murine IHC except CD31. As immunohistochemical controls, the primary antibodies were omitted and isotype controls specific to the primary antibody were also run.

Positive control tissue for Lyve-1, VEGFR-3, CD20 and CD3 were human lung, placenta, Burkitt's lymphoma and tonsil respectively.

Formalin fixed sections required antigen retrieval carried out with 5mls of vector antigen solution (Vector, UK) in 500mls of deionised water at high temperature for 15 minutes.

Exceptions included:

- 1) iNOS/LYVE-1/Ki-67: High temperature retrieval for 15 minutes in citrate buffer PH 6.
 - 2) CD3: Pressure cooker for 2 minutes in 1000mls antigen retrieval solution.
 - 3) CD31: Protease 12.5mg/100ml PBS preheated to 37⁰C. Sections incubated for 20mins at 37⁰C.
- CD31 – DAB was applied for 10mins and intensified in copper enhancement solution (6.66g cupric sulphate and 11.88g sodium chloride dissolved in 1000ml deionised water) applied for 5 minutes

Table 2- 1 Antibodies used for immunohistochemical staining

Antigen/cells	Primary antibody	Secondary antibody (all biotinylated)	Isotype control	Colour detection
Mϕ human tissue	Monoclonal mouse anti human CD68 (DAKO, Denmark) 1/400, 30mins RT	Rabbit anti mouse (DAKO, Denmark) 1/300 30mins RT	Mouse serum (sigma UK) protein concentration 47.9mg/ml	DAB colour reagent (DAKO Cytomation)
Mϕ murine tissue	Rat anti mouse F4/80 (Caltag) 1/100 1hr RT	Rabbit anti rat (Vector) 1/300 30mins RT	Rat IgG (Sigma UK)	DAB colour reagent (DAKO Cytomation)
iNOS	Polyclonal rabbit anti human iNOS (ABCAM, UK), 1/50 overnight 4°C	Goat anti rabbit (DAKO Denmark). 1/300. RT 30mins	Rabbit polyclonal IgG isotype control (abcam) prediluted	DAB colour reagent (DAKO Cytomation)
T cells Murine & human	Monoclonal rat anti CD3 (Vector,UK) 1/100 1hr RT	Rabbit anti Rat Vector, UK) 1/100 30 mins RT	Rat IgG (Sigma UK) protein concentration 11.3mg/ml)	DAB colour reagent (DAKO Cytomation)
B cells Murine	Rat anti mouse B220(PharMingen) 1/50 30mins RT	None required	Rat IgG2a (abcam,UK)	DAB colour reagent (DAKO Cytomation)
B cells Human	Mouse anti human CD20 (DAKO, Denmark) 1/100 30mins RT	Rabbit anti mouse (DAKO, Denmark) 1/300 30mins RT	Mouse serum (sigma UK)	DAB colour reagent (DAKO Cytomation)
VEGF-C Human	Polyclonal goat anti human VEGF-C (Bioscience, USA). 1/20 4°C overnight	Rabbit antigoat (DAKO, Denmark)1/300 30mins RT	Goat serum (0.5mg/ml) (sigma UK) 1:400	DAB colour reagent (DAKO Cytomation)
VEGF-A Human & Murine	Polyclonal chicken anti VEGF-A (abcam, UK) 1/100 2hrs RT	Goat anti chicken (Vector) 1/300 30mins RT	Chicken serum (abcam, UK) (7mg/ml) 1:700	DAB colour reagent (DAKO Cytomation)
Proliferation marker Ki67	Polyclonal rat anti Ki67 (abcam,UK) 1/25 30mins RT	Rabbit anti rat (DAKO, Denmark) 1/300 30mins RT	Rat IgG2a (abcam, UK)	DAB colour reagent (DAKO Cytomation)

Antigen/cells	Primary antibody	Secondary antibody (all biotinylated)	Isotype control	Colour detection
Endothelium Murine	Monoclonal rat anti CD31 (BD Pharmingen) 1/10 4°C overnight	Rabbit anti-Rat (DAKO, Denmark) 1/300 30mins at RT	Rat IgG (Sigma UK)	DAB colour reagent (DAKO Cytomation)
Endothelium Human	Monoclonal mouse anti human CD34 (serotec) 1/50 4°C overnight	Rabbit anti mouse (DAKO, Denmark) 1/300 30mins at RT	Mouse serum (Sigma UK)	DAB colour reagent
Lymphatic Endothelium human	Monoclonal mouse anti human podoplanin (abcam, UK), 1/100 1hr RT	Rabbit anti mouse (DAKO Cytomation) 1/300 RT 30 mins	Mouse serum (Sigma UK)	DAB colour reagent (DAKO Cytomation) or VIP very intense purple (Vector) for double stain
Lymphatic Endothelium murine	Monoclonal hamster anti mouse podoplanin (abcam UK) 1/3500 30mins RT	Goat antihamster (vector, UK) 1/300 30mins RT	Syrian hamster IgG Isotype control (abcam, UK)	DAB colour reagent (DAKO Cytomation)
Lymphatic Endothelium human	Mouse anti human D240(abcam, UK) Prediluted, 90mins RT	Rabbit antimouse (DAKO, Denmark) 1/300 RT 30mins	Mouse serum (abcam) IgG concentration 250mg/ml	DAB colour reagent (DAKO Cytomation) or Fuchsin red (Vector,UK) for double stain
Lymphatic Endothelium human	Polyclonal rabbit anti human LYVE-1 (abcam, UK), 1/100 overnight 4°C	Goat anti Rabbit (DAKO, Denmark) 1/300 30mins RT	Rabbit polyclonal IgG isotype control (abcam) prediluted	DAB colour reagent (DAKO Cytomation)
Lymphatic Endothelium human	Rabbit anti human VEGFR-3 (abcam, UK) prediluted, 1hr RT	Goat anti Rabbit (DAKO, Denmark) 1/300 30mins RT	Rabbit polyclonal IgG isotype control (abcam) prediluted	DAB colour reagent (DAKO Cytomation)

2.1.2.2 Double labelling :

This technique was used in order to look for co-localisation between lymphatics and M ϕ . The following flow diagram demonstrates IHC double labelling:

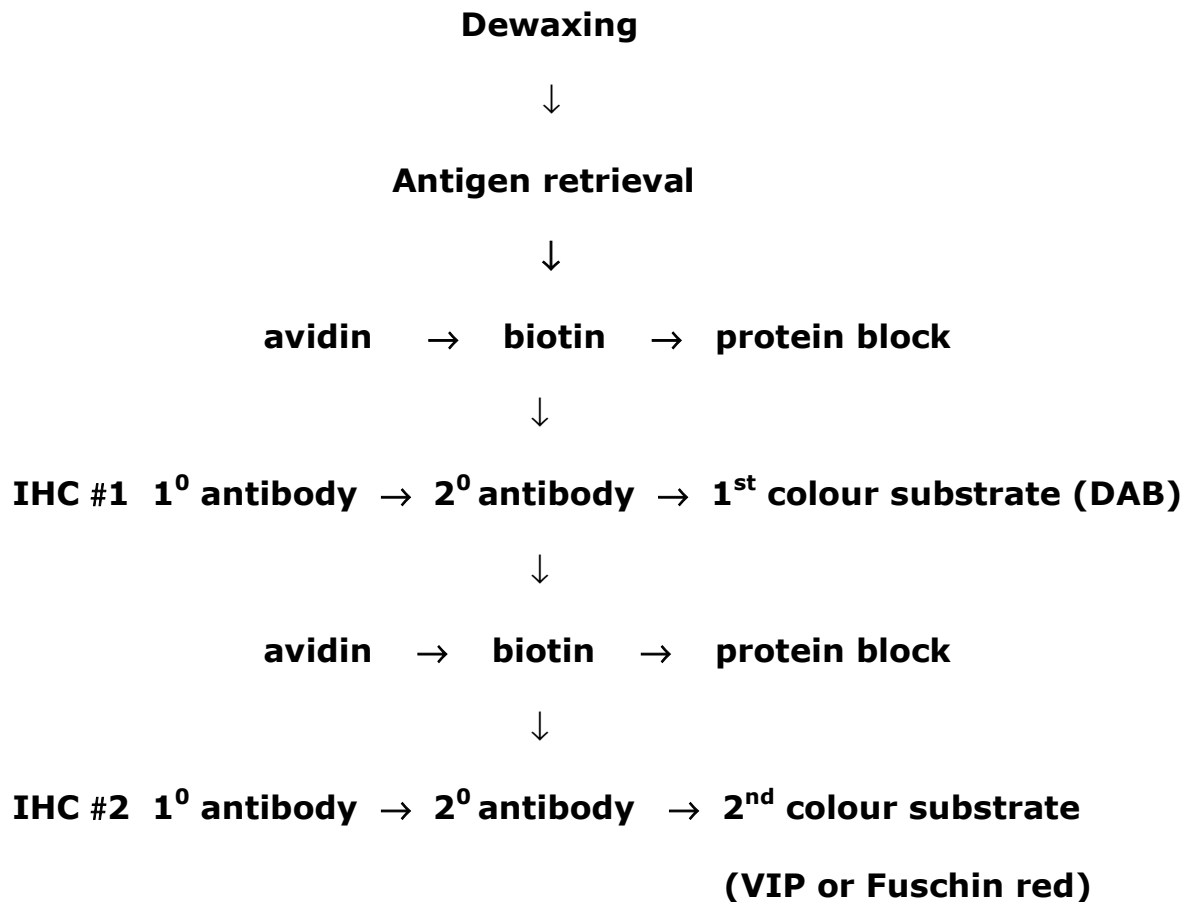


Figure 2- 3 Immunohistochemistry double labelling

2.1.2.3 IHC analysis of tissue sections

Human tissue

1. *M ϕ Infiltration (CD68):* Methods for quantifying CD68 immunostaining in human tissue was discussed with Dr C Bellamy (renal pathologist) who recommended a semi quantitative scoring system graded 1-7 (zero-severe) as follows:

1= zero

2= minimal

3=mild

4 =mild-moderate

5=moderate

6=moderate-severe

7=severe

A total of 20 fields (10 fields in the cortex and 10 fields in the medulla) were examined at x200 magnification and graded 1-7 for CD68 positive cell infiltration.

2. *Lymphatic Vessels (Podoplanin (D240) stain)*

20 fields at X200 magnification were analysed (10 fields in the interstitium and 10 fields in the perivascular region). The mean number of podoplanin or D240+ve vessels per field in the interstitium and perivascular region were determined (Kerjaschki et al., 2004). A lymphatic vessel was defined as a podoplanin positive structure with a visible lumen

Murine tissue

1. Leukocyte infiltration (*M ϕ* – F4/80, B cells – B220, T cells – CD3).

For each section 10-15, fields including both cortex and medulla were examined and pictures were taken at x200 magnification. For quantification areas with positive staining for F4/80, B220 and CD3 were measured using computer image analysis (website rsb.info.nih.gov/ij/). In each Image J field the positive areas were identified and expressed as a percentage of the total field area. (Kipari et al., 2006; Ohashi et al., 2002) (fig 2-4).

2. Lymphatic Vessels (Podoplanin)

Tissue sections were examined (x400 magnification) and the number of fully formed podoplanin positive lymphatics with a visible lumen per intra renal artery were counted.

3. iNOS stain. Using a 25-point Chalkley graticle the number of iNOS positive interstitial cells and iNOS positive tubules were counted at x400 magnification. 10 cortical fields were examined.

4. Peritubular capillaries (CD31)

In each section 10-15 randomly selected fields including both cortex and medulla were examined and pictures were taken at x400 magnification. Image J software was then used to count the number

of CD31 positive peritubular capillaries (PTC). For the exclusion of the effects of tubular dilation and atrophy on measured values, the number of tubules in each field was also counted and the number of PTC were expressed per 100 tubules (Ohashi et al., 2002) (website rsb.info.nih.gov/ij/).

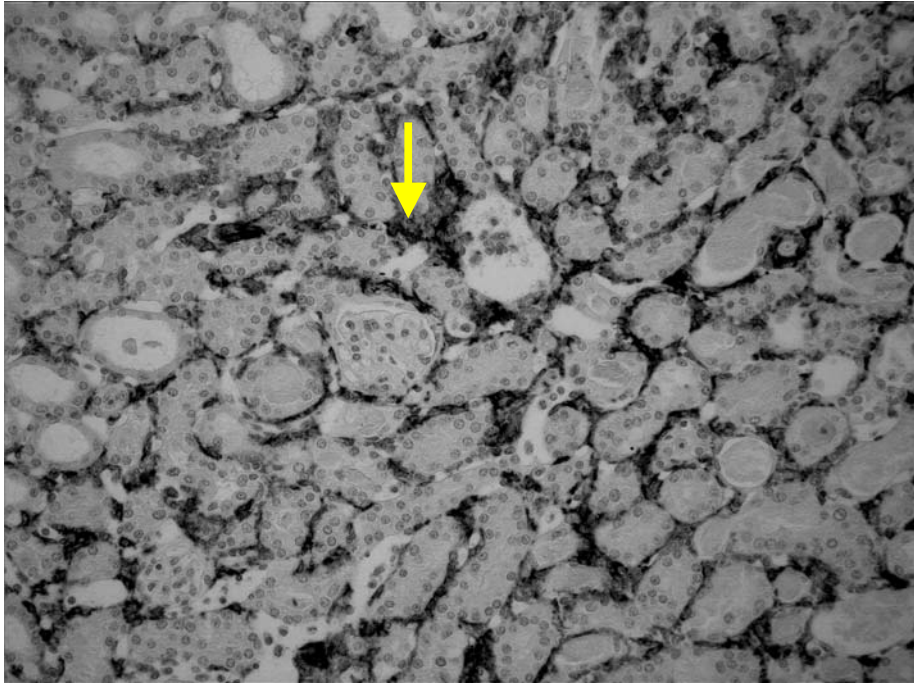
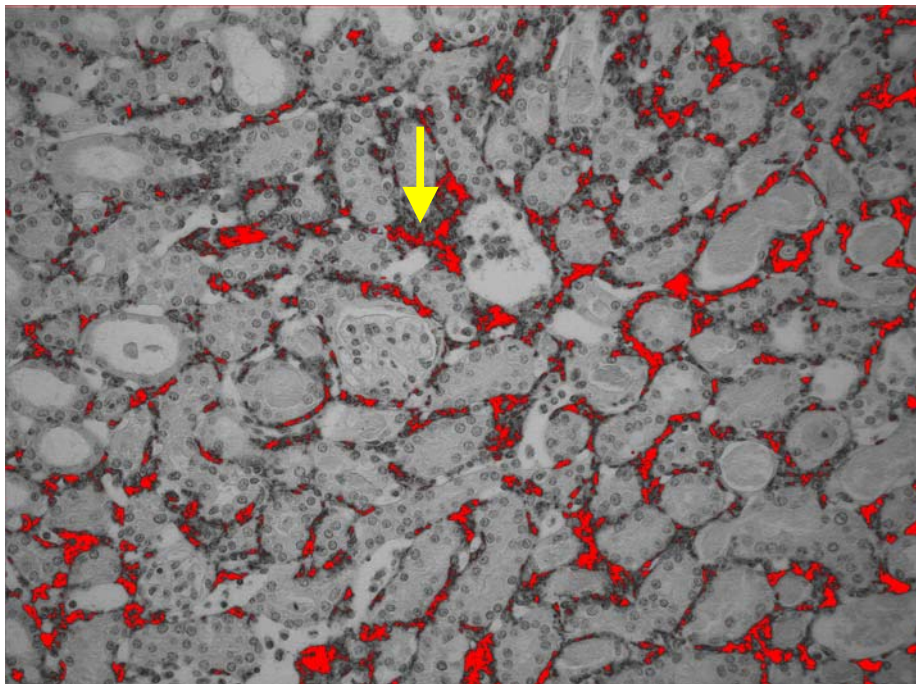
A**B**

Figure 2- 4 Image J analysis of F4/80 stain.

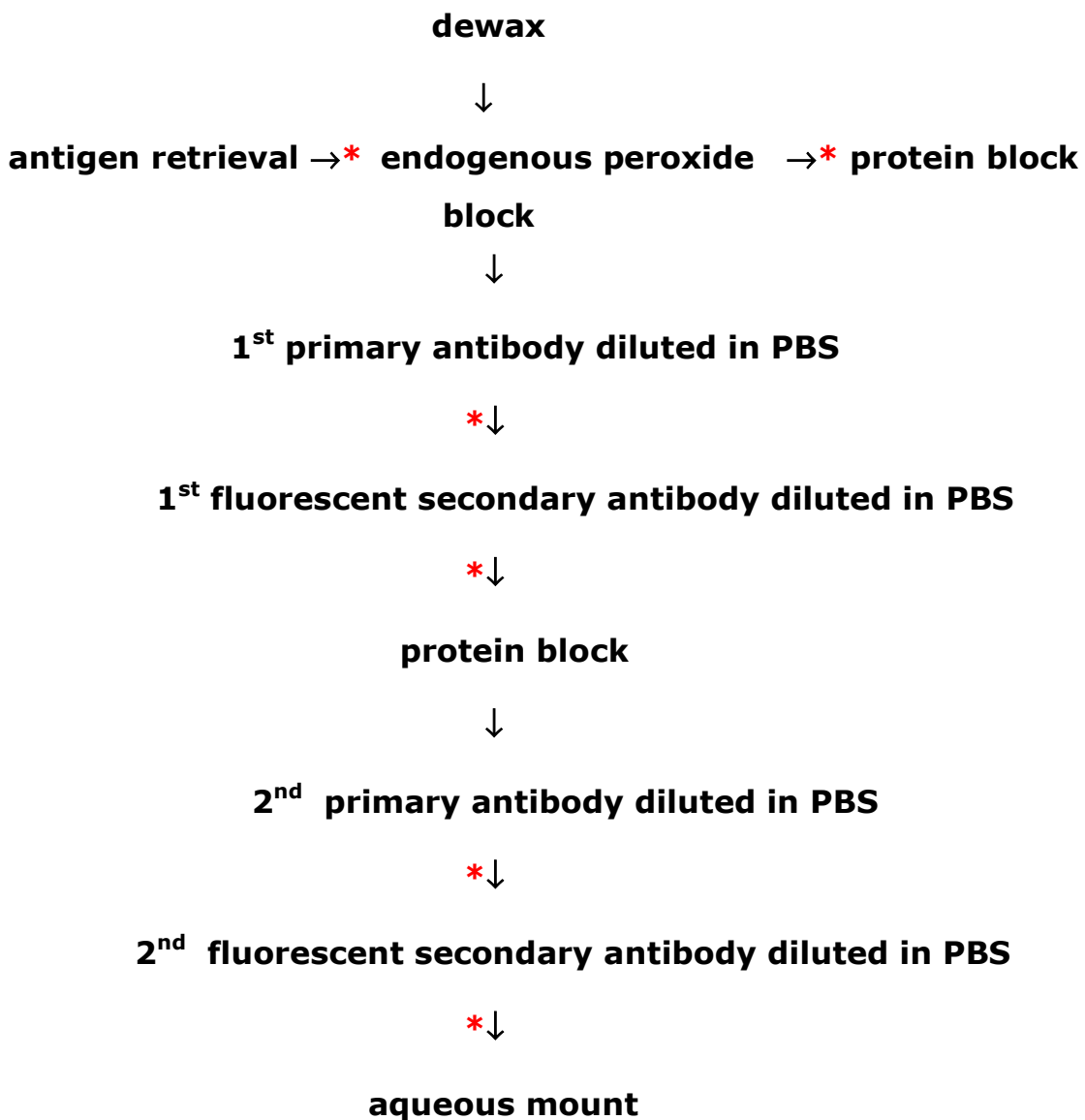
Murine allograft tissue. In each field the positive areas were identified (example arrowed) and expressed as a percentage of the total field area. **(A)** computer picture. **(B)** pixel image with threshold set.

2.1.3 Immunofluorescence (IF)

Immunofluorescence was used on the human sections to investigate co-localisation between two substances within the same cell, which would not be seen using IHC double staining.

2.1.3.1 Method for IF

The following flow chart describes the method for immunofluorescence staining:



* = washing steps

Figure 2- 5 Method for immunofluorescent staining

*Each washing step was carried out in TBS x1, which is 100mls of x10 TBS diluted in 900mls of saline. X10 TBS was made up by dissolving 30.25g Tris Bas in 400ml-distilled water and adjusting PH to 7.6.

A black box was used following addition of the fluorescent secondary to ensure that staining did not fade.

The following controls were included: isotype control for primary antibody, secondary antibody alone with omission of primary antibody and no immunostaining (control for tissue autofluoresence).

Table 2- 2 Antibodies used for IF

Cell/Antigen	Primary antibody	Secondary antibody ALEXA (Molecular Probes, UK)	Isotype control
Mϕ	Monoclonal mouse anti human CD68 (DAKO, Denmark) 1/400, 30mins RT	Rabbit anti mouse-488 or 647 1/200 30mins RT	Mouse serum (Sigma UK)
iNOS	Polyclonal rabbit anti human iNOS (ABCAM, UK), 1/50 overnight 4°C	Goat anti rabbit-647 1/200 30mins RT	Rabbit polyclonal IgG isotype control (abcam) prediluted
T cells	Monoclonal rat anti human CD3 (Vector,UK) 1/100 1hr RT	Donkey anti rat-488 or 594 1/200, 30mins RT	Rat IgG (Sigma UK)
B cells	Monoclonal mouse anti human CD20 (DAKO, Denmark) 1/100, 30mins RT	Rabbit anti mouse-647 30mins RT	Mouse IgG2a κ (BD Pharmingen)
VEGF-C	Polyclonal chicken anti human VEGF-C (Bioscience, USA). 1/20 4°C overnight	Rabbit anti goat-647 30mins RT	Chicken serum (Abcam UK)
VEGF-A	VEGF-A chicken polyclonal (abcam, UK) 1/100 2hrs RT	Goat anti chicken-488 30mins, RT	Chicken serum (Abcam UK)

2.1.3.2 *Microscopes used for IF studies*

The microscopes used for analysis were the ZEISS LSM5 10 META and LEICA. Confocal laser scanning is a system based on an inverted motorised axiovert 200M stand with a LSM510 META scan head. It provides 6 laser lines for fluorochrome excitation - 458,477,488,514,543,633nm.

2.2 Cell culture

2.2.1 General reagents

Tissue culture reagents were purchased from Life Technologies (Paisley, Scotland, UK). Tissue culture plastics were obtained from Costar (Loughborough, Leicestershire, UK) and Falcon (Runcorn, Cheshire, UK). Cytokines were purchased from R&D Systems (Abingdon, UK) and Peprotech EC Ltd (London, UK).

L-N6- (1-iminoethyl)-lysine (L-NIL) and the control inactive isomer D-N6- (1-iminoethyl)-lysine (D-NIL) were purchased from Fluorochem Ltd (Old Glossop, Derbyshire, UK). All other reagents were from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) unless otherwise stated.

2.2.2 Murine cardiac endothelial cell (MCEC-1)

2.2.2.1 Description

MCEC-1 is a well established cell line isolated from transgenic mice harbouring the temperature sensitive simian virus 40 large Tag (tsA58 Tag) control gene under the H-2kb class 1 promoter. These cells proliferate at 33⁰C and behave like primary cells at 37⁰C (Lidington et al., 2002) (Gift from Justin Mason, Imperial college London).

2.2.2.2 *MCEC-1 cell*

All work carried out in the tissue culture hood employed general sterile tissue culture techniques. The MCEC -1 cells were cultured on gelatin coated T75 flasks (1% gelatin) in full MCEC-1 media – Dulbecco's Modified eagle's Medium (DMEM) and 10% Fetal calf serum (FCS) (GIBCO BRL) with penicillin (100U/ml), streptomycin (100µg/ml), 2mM L-glutamine (GIBCO BRL) and endothelial cell growth factor (ECGF) (10U/ml) (Leo Laboratories Ltd, UK). These MCEC-1 cells were routinely cultured at 33⁰C and maintained in culture by passaging cells when they were 70-80% confluent. The MCEC-1 cells adopted a cobble stone appearance when confluent. Initial stock cells were passage 1. Passages 5-9 were used in all the experiments, which were performed at 37⁰C.

2.2.2.3 *Thawing and passaging cells*

A cryovial of frozen MCEC-1 cells that had been stored in liquid nitrogen (-196⁰C) was thawed by agitating the vial by hand in a 37⁰C water bath. The contents were then transferred into a 15ml falcon tube containing prewarmed full MCEC-1 medium but no ECGF. This was spun for 5 minutes at 1000rpm (210g). The medium was aspirated off and the pellet of cells resuspended in 10ml of medium plus ECGF. This was transferred to pre-prepared T75 flasks containing 5mls of warmed media. New T75 flasks were pre-coated with 1% gelatin and incubated at 37⁰C for 30mins. This was then

removed by aspiration and replaced by 5mls of medium. When >70% confluent the cells were split 1:3 or 1:10 for slower growth according to when the cells were required for experiments.

To release the endothelial cells the monolayer was washed twice in 10mls of PBS without calcium or magnesium. 1ml of trypsin/EDTA was added and incubated for 2-3 min at 37°C. The flask was firmly tapped to release cells and detachment was confirmed by examination under the microscope. The cells were resuspended in 10ml of medium (no ECGF), transferred into 15ml falcon tube and centrifuged for 5mins at 1000rpm (210g). The medium was aspirated off leaving a pellet of MCEC-1 cells at the bottom. This pellet was resuspended in full MCEC medium with ECGF and added to the preprepared flask.

2.2.2.4 Freezing and storage of cells

The cells were detached as described above and resuspended in 2mls of 10% dimethyl sulphoxide (DMSO) and FCS (90%). Two 1ml containers were filled and stored at -70°C and then transferred into liquid nitrogen.

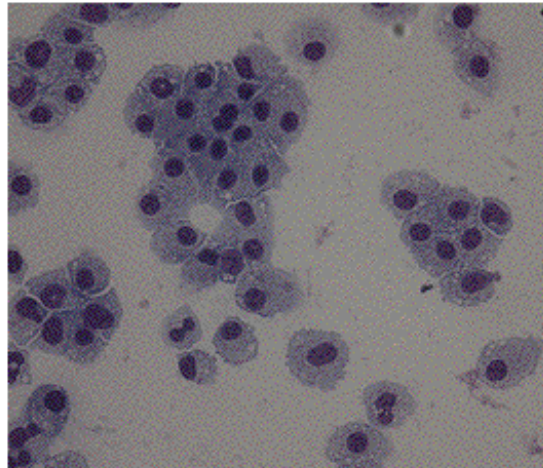
2.2.3 Culture of primary bone marrow derived macrophages (BMDM)

2.2.3.1 Preparation BMDM

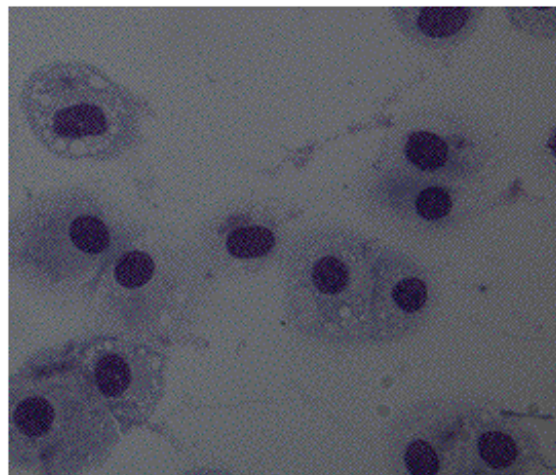
BMDM were prepared from C57Bl/6 female mice as described previously (Duffield et al., 2000; Kipari et al., 2006; Kipari and Hughes, 2002). Briefly, bone marrow was isolated from the femurs by standard sterile techniques and matured for 7 days in 60ml non adherent teflon pots (R Vetter, Germany) in DMEM/F12 medium with 10% FCS, penicillin (100U/ml), streptomycin (100ug/ml) and 20% L929 cell conditioned medium as a source of M ϕ colony stimulating factor (M-CSF) to stimulate differentiation and maturation of M ϕ . 10mls of the media was replenished every second day with fresh media.

Characterisation of BMDM: Maturation and purity of BMDM at day 7 were checked by performing a cytopspin followed by Diff Quik staining. 100 μ l of cells in suspension were added to a cytopspin holder containing a glass slide, piece of filter paper and a plastic well. The cells were then spun for 3 minutes at 300g. The cells attached to the glass slide and were then fixed with 100% methanol for 2mins, followed by staining in the Diff quik red solution for 1 minute and Diff quik Blue solution for 1 minute. All steps were performed at room temperature. The slides were rinsed with distilled water, left to air dry over night and finally cover slipped and mounted. Cytopspins were examined under a microscope looking for M ϕ morphology (fig

2-5). Phenotyping using flow cytometry has previously been carried out in our lab on these M ϕ populations.



x200



x400

Figure 2- 6 Characterisation of BMDM using cytopsin and Diff Quick staining of BMDM in suspension at day 7 maturation

2.2.3.2 *Macrophage supernatant harvesting*

250,000 M ϕ in 24-well plates were cultured in 500 μ l of full DMEM/F12 medium for 24 hours in the presence or absence of IFN γ and LPS. After 24 hours, the supernatants were collected and centrifuged (4 $^{\circ}$ C , 5minutes, 4000g) to remove any cell debris.

This supernatant was then used for:

1. Co-culture with MCEC-1 cells. The M ϕ supernatant was added to Tracker-green labelled MCEC-1 cells (50,000/well) and incubated for 24h at 37 $^{\circ}$ C.
2. Griess Assay for analysis of nitrite concentration and specific ELISA for analysis of TNF α concentration (section 2.3 and 2.4)

2.2.4 Co-culture experiments

The interaction between BMDM with MCEC-1 cells was studied using a co-culture assay well established in our laboratory (Duffield et al 2000, Kipari et al 2006) (fig 2-7)

Target cell: Murine cardiac endothelial cells (MCEC-1)

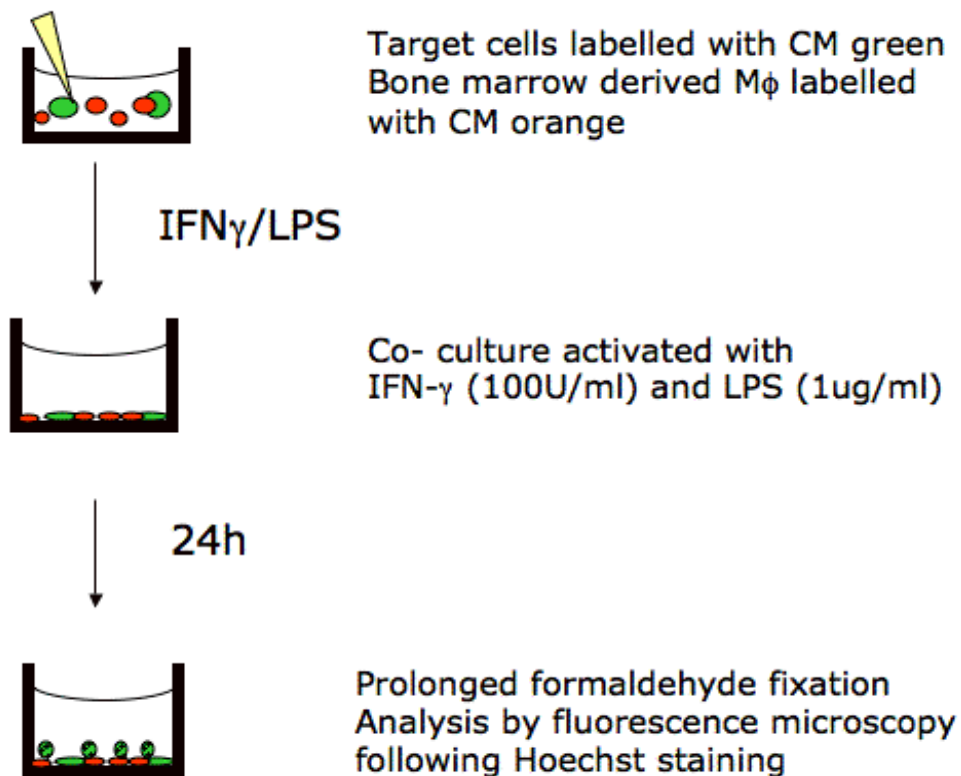


Figure 2- 7 In vitro co-culture assay of interaction between murine BMDM and MCEC-1 cells.

2.2.4.1 *Direct co-culture between BMDM and MCEC-1*

MCEC-1 cells were prelabelled with fluorescent cell tracker green: MCEC-1 cells were washed in serum free MCEC-1 medium and incubated for 45 minutes with serum free medium containing CM-green tracker dye at a concentration of 5ng/ml (Molecular Probes, Eugene, OR, USA). Cells were washed with medium containing serum to quench unbound tracker dye, then trypsinised and 1.5×10^4 cells were added to 48 well plates to cover 70- 80% of the well surface area. Cells were allowed to adhere for 2-4 hours. M ϕ were incubated for 30 minutes with fluorescent cell tracker orange at a concentration of 5ng/ml. M ϕ were added to the MCEC-1 cells at a ratio of 2:1 M ϕ :MCEC-1 cell as this ratio had been used previously (Kipari et al., 2006). Co-cultures were incubated for 3 hours before selected wells were activated with LPS (1ug/ml) and murine IFN γ (100U/ml). Non-activated wells were exposed to medium alone.

The four experimental conditions were:

- MCEC-1 cells alone (control well)
- MCEC-1 cells activated with IFN γ /LPS
- Co-culture non-activated in medium without cytokines
- Co-culture activated with IFN γ /LPS.

After 24 hours incubation the undisturbed wells underwent fixation with formaldehyde (4% final concentration) in order to ensure retention of apoptotic cells (Duffield et al., 2000; Kipari et al., 2006). Staining with Hoechst 33342 at 1ug/ml was then carried out for 15

minutes. Each experimental condition was carried out on three separate occasions. M ϕ were derived from at least three different animals.

2.2.4.2 Assessment of apoptotic cell retention in fixed co-cultures

Previous work indicated that formaldehyde fixation of the co-culture plates allowed fixation and retention of apoptotic mesangial and tubular cells. However apoptotic and necrotic cells do have the propensity to detach and float in the culture medium and therefore there was a risk of losing these apoptotic cells before analysis. For this reason after 48 hours of fixation the formaldehyde solution containing any unattached cells termed 'floaters' was collected in FACS tubes. This was washed with PBS and the washing fraction was also collected in the same tube. Centrifuge at 300g for 5 minutes at 4⁰C was then carried out to remove formaldehyde and the supernatant was removed. The floaters resuspended in fresh PBS were then analysed using flow cytometry.

Results showed that only a negligible proportion of the cells were lost, <1% of total cell number and so it was decided not to collect floaters routinely in future experiments.

2.2.4.3 Assessment of MCEC-1 cell apoptosis and proliferation by fluorescent microscopy

Analysis was carried out using inverted fluorescent microscopy. Five non-overlapping fields at high power (x400) were blindly chosen. Apoptotic MCEC-1 cells were identified by their green condensed cytoplasm and pyknotic nucleus. Mitotic MCEC-1 cells were recognised by their chromatin pattern and the presence of daughter cells. The total number of MCEC-1 cells were counted and expressed as a percentage of the control group (MCEC alone), which was depicted as 100%.

2.2.4.4 *Video microscopy*

Video microscopy was carried out using a NIKON microscope. (Eclipse TE2000-U). A co-culture experiment was set up as described above and activated co-cultures wells were analysed over an 18 hour time course with pictures being taken every 5 minutes. (see attached CD-ROM)

2.3 Griess Assay

This reaction measures nitrite concentration in fluids. Nitrite is a stable and non volatile breakdown product of NO. The Griess assay was used to analyse murine M ϕ supernatant and urine from murine transplant recipients. All reagents are from Promega Griess Reagent system unless otherwise stated.

2.3.1 Method:

50 μ l of M ϕ supernatant was added to the wells of a 96 well plate in duplicate. Following addition of 50 μ l of sulphanilamide solution (1% sulphanilamide in 5% phosphoric acid) to all experimental samples and the wells containing the dilution series for the standard curve (Nitrite standard, 0.1M sodium nitrite in H₂O), the plate was incubated at room temperature in the dark for 10 minutes. 50 μ l of 0.1% N-1-Napthylethylenediamine dihydrochloride (NED solution) was added to all wells, and the plate was then incubated at room temperature in the dark for 10 minutes. Absorbance was measured in a plate reader with a filter between 520-550nm.

A nitrite standard reference curve was produced by plotting the average absorbance value of each concentration of the nitrite standard against nitrite concentration.

The average absorbance value of each experimental sample was calculated and its nitrite concentration was determined by comparison to the nitrite standard reference curve.

2.4 Enzyme linked immunosorbent assay (ELISA)

ELISA was used to determine the TNF α concentration in murine M ϕ supernatant.

2.4.1 Method

ELISA was used to quantify murine TNF α from M ϕ supernatants (N=3 with M ϕ from 3 different mice) according to the manufacturer's instructions (R&D Systems, Abington, Oxfordshire, UK). Briefly, the goat anti-mouse TNF α capture antibody was diluted in PBS (1/180) and used to coat a 96 well plate (100 μ l/well). The plates were sealed and incubated overnight at room temperature. The capture antibody was removed and the wells were washed 3 times using ELISA wash buffer (PBS tablets) diluted in distilled water (1 tablet/100ml water) with 0.05% Tween-20, PH 7.2-7.4). 300 μ l of blocking buffer was applied to each well for 1 hour at room temperature (1% BSA in PBS, pH 7.2-7.4). Blocking buffer was aspirated and the wells washed 3 times with ELISA wash buffer.

100 μ l of either standard in reagent diluent (1% BSA in PBS, pH 7.2-7.4) or sample was applied to each well. Both standards and samples were run in duplicate. The plates were sealed and incubated at room temperature for 2 hours.

Wells were washed 3 times with ELISA wash buffer and 100 μ l of the biotinylated goat anti-mouse TNF α detection antibody diluted in reagent diluent (1/180) was added to each well. Following a 2 hour incubation, the detection antibody was removed and the wells

washed with ELISA wash buffer. 100 μ l of strepavidin-HRP was applied to each well (diluted in reagent diluent 1/200). The plate was covered and incubated for 20 minutes at room temperature. Further washing with ELISA wash buffer was followed by the addition of 100 μ l of substrate solution (1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (Tetramethylbenzidine)) to each well and the plates were incubated for a further 20 minutes and protected from light. The reaction was then stopped by adding 50 μ l of stop solution (2N H_2SO_4) to each well.

The optical density of each well was evaluated using a microplate reader (Dynatech Laboratories, USA) set to a wavelength of 450nm with the wave length correction set to 570nm.

2.5 Flow Cytometric Analysis of Cells

2.5.1 Fas Ligand expression by BMDM

Mature BMDM were grown on 12 well plates (500,000/well) as previously described. After incubation at 37°C overnight M ϕ were detached from the tissue culture dish by scraping. Cells were blocked with 1% mouse serum and PBS for 15 minutes at 4°C and incubated with FITC conjugated rat monoclonal antibody to Fas ligand (Abcam 1: 100) or isotype control FITC conjugated Rat IgG2a (Bioscience, 1:200) for 45 minutes at 4°C in the dark. The positive control used was the cell line K5625 which is an erythroleukaemic cell line which has been transfected with Fas-L (Smith et al., 1998).

2.6 Lymphatic endothelial cell identification & isolation

2.6.1 Cells examined

In an attempt to isolate a lymphatic endothelial cell population *in vitro* the following cells were studied:

- Human umbilical vein endothelial primary cells (HUVCs) (Promocell, UK)
- Human dermal microvascular primary endothelial cells (HUDMECs) (Promocell, UK)
- Human dermal microvascular endothelial cell line (HUDMECs). (Gift from Centre for Disease Control and Prevention, Atlanta, Georgia)

2.6.2 Method for passaging, freezing & thawing cells

2.6.2.1 Passaging cells

Cells were grown to 80% confluence in T25 flasks at 37⁰C.

Human dermal microvascular endothelial cell HDMEC (Cell Line):

The medium was aspirated and the cell monolayer was washed twice with PBS without calcium or magnesium. 0.5mls of Trypsin/EDTA was added and the cells were allowed to detach at 37⁰C for 3-5 minutes with detachment checked by microscopy. 4.5mls of Clonetics endothelial basal medium (CAMBREX) with 10% FCS, 1mg/ml hydrocortisone (Sigma, UK), 10ng/ml endothelial growth factor (BD), penicillin and streptomycin was added to the cells which

were then transferred into a 15ml falcon tube. Cells were counted using a haemocytometer and inoculated into new T25 flasks containing 5 mls of fresh full medium.

Human dermal microvascular endothelial cells HDMEC (Primary Cells): The media was replaced with 5mls HepesBSS containing 30mM Hepes, D-Glucose, sodium chloride, sodium phosphate, Phenol red (Promocell). This was then removed after 30 seconds and the monolayer was covered with 2.5mls trypsin/EDTA. When the cells were completely detached 2.5ml of trypsin neutralisation solution (Promocell) was added. The cells were then removed from the flask and centrifuged at 220g for 5 minutes. The clear supernatant was aspirated off and the pellet resuspended in 1ml of endothelial cell growth medium with FCS 2%, Endothelial growth factor 0.1ng/ml, hydrocortisone 1ug/ml, basic fibroblast growth factor 1ng/ml and endothelial cell growth stimulating hormone (ECGS/H) 0.4% (Promocell) before placing the seeded cells in a T25 flask containing 5mls full medium at 37°C.

2.6.2.2 *Freezing storage and thawing of cells*

The cells were detached as described above followed by centrifugation at 220g for 5 minutes at 20⁰C. The medium was then aspirated off and the pellet resuspended in FBS and 10% DMSO for the cell line or Cryo-SFM (promocell) for the primary cells.

The cells were thawed in a 37⁰C water bath and then added to 10mls of full media in a 15ml falcon tube. This was centrifuged at 250g for 5 minutes and the pellet was resuspended in 10mls of fresh medium, which was finally placed in a T25 flask and incubated at 37⁰C.

2.6.3 Methods for phenotyping cells

2.6.3.1 Flow cytometry

Method

HDMECs cultured at 37°C were grown to 80-90% confluence, detached from the T25 flask as previously described and transferred to a 15ml falcon tube. This suspension was then spun at 220g for 4-5 minutes and the medium was aspirated off leaving the pellet of cells at the bottom.

On ice: These cells were washed in PBS, centrifuged at 220g for 4 minutes followed by a blocking step: 100ul of PBS +/- with 10% FCS per tube and left on ice for 10minutes.

The primary antibody diluted in PBS and 10% FCS was then added and the cells were left on ice for 45 minutes. Cells were washed with FACS buffer (0.5% BSA in PBS) before incubation with the secondary antibody (diluted in PBS and 10% FCS) for 45 minutes.

For double staining a further wash was performed after incubating with the secondary antibody before the second primary antibody was added. A final wash in ice cold PBS was then carried out before flow cytometric analysis of the cells. Controls consisted of:

- Unstained cells.
- Cells stained with the secondary antibody alone.
- Cells stained with the isotype control antibody for the primary antibody.

2.6.3.2 Immunofluorescence

Method

Cells were plated on cover slips (75,000 cells/cover slip) and placed in a 24 well plate in full medium overnight. Washing of the cells in PBS with 0.1% BSA was then followed by fixation in ice-cold methanol: acetone 1:1 for 2 minutes. The cover slips were left to air dry for 20 minutes before the primary antibody (diluted in PBS with 0.1% BSA) was added and incubated at room temperature for 1 hour. Three washes in X1 TBS preceded incubation of the cells with the secondary antibody (diluted in PBS with 0.1% BSA) conjugated to the fluorochrome for 30 minutes at room temperature. Further washing in x1 TBS followed by a wash in distilled H₂O was carried out before mounting the slides in DAPI mounting medium.

Table 2- 3: Antibodies used for phenotyping cells

Antigen/Cell	Primary Antibody	Secondary antibody	Isotype control
Endothelium CD31	Preconjugated mouse anti-human CD31 (Serotec) 1/75 30mins	Not required	Mouse IgG Serum (Caltag)
Lymphatic Endothelium Podoplanin	Polyclonal mouse antihuman podoplanin (Abcam,UK) 1/100 1hr	Rabbit antimouse ALEXA 488 (molecular probes, UK) 1/200 30mins	Mouse IgG1 serum (Caltag)
Lymphatic Endothelium VEGFR-3	rabbit antihuman VEGFR-3 (Abcam, UK) prediluted 1hr	Goat anti rabbit ALEXA 488 (molecular probes, UK) 1/200 30mins	Rabbit IgG isotype control (Abcam)

2.6.3.3 *Electron microscopy studies*

Approximately 100,000 cells were plated on thermanox plastic cover slips in 24 well plates with 300µl of medium. When confluent the medium was aspirated off and the cells washed with PBS before being fixed with 3% glutaraldehyde in 0.1M sodium cacodylate buffer and placed in the fridge.

Sections were then prepared by Steve Martin at the College of Veterinary Medicine, Edinburgh and transmission electron microscopy (TEM) was performed on the HDMECS cell line mounted on cover slips.

2.6.4 Magnetic bead sorting of HDMEC cell line

Method:

HDMECs were grown to 80% confluence, detached from the T25 flask and the cell pellet isolated as previously described.

All steps were now carried out at 4⁰C. The cell pellet was resuspended in 200µl of MACS buffer (200mls PBS with 1g BSA and 800ul EDTA (Sigma, UK)), and incubated with 3µl of the human anti podoplanin antibody (Abcam, UK) for 20 minutes at 4⁰C. The cells were then resuspended in MACs buffer and then incubated with 10µl of goat anti-mouse IgG microbeads (Miltenyi Biotec, UK) for 15 minutes at 4⁰C. Following a further wash, magnetic separation was carried out. The chilled separation column (Miltenyi Biotec, UK) was placed in the magnetic field of a MACS separator and prepared by rinsing with 3ml of buffer. The cell suspension was placed into the column and the unlabeled cells were collected in a 50ml falcon tube as they passed through - this was negative selection for podoplanin negative cells. The column was then removed from the separator and placed in a 15ml falcon tube and 5ml of buffer was pipetted into the column - these were the positively selected podoplanin positive cells. The cells were spun and resuspended in full medium. 2mls of cell suspension were plated on each well of a 6 well plate.

2.7 Statistical analysis

All results are presented as mean \pm SEM or median plus interquartile range. Statistical analysis was performed using GraphPad Prism 3.02/InStat 1.1 (GraphPad software, San Diego, CA USA). The Students t-test was employed for comparisons involving two groups and statistical differences among multiple groups of data were assessed by Mann-Whitney test. Results are considered significant at $p < 0.05$.

Chapter 3.

Analysis of the lymphatic and vascular endothelium in human chronic allograft nephropathy

3.1 Introduction

In the studies outlined in this chapter immunohistochemical and immunofluorescent studies were undertaken on human renal tissue to investigate changes in the lymphatic system and infiltration of inflammatory cells occurring in the context of CAN.

Previous work by my predecessor David Mitchell using CD31 immunostaining of human renal tissue demonstrated a significant reduction in microvascular density in CAN tissue compared to control kidney tissue. In addition evaluation of endothelial cell cycle activity assessed with dual immunostaining with MIB-1 and CD31 demonstrated significant endothelial cell proliferation in CAN tissue with very few MIB-1 positive endothelial cells in control kidney (Adair et al., 2007). Further studies went on to investigate the changes in lymphatic vessel density, inflammatory infiltrate and VEGF-A and -C expression in these tissues.

3.2 Patient Information

Regional ethical approval was obtained. Pathology reports from transplant nephrectomies performed at the Royal Infirmary of Edinburgh were reviewed by a pathologist looking for changes consistent with CAN.

CAN tissue was available from 29 patients who underwent a transplant nephrectomy (age range 14-74, mean 40) and were diagnosed with CAN according to the Banff criteria (Racusen et al., 1999). In our centre maintenance immunosuppression consists of triple therapy: calcineurin inhibitor, an antiproliferative agent e.g. azathioprine, and prednisolone. At allograft nephrectomy, the majority of patients (n=19) had stopped immunosuppressive therapy and all had restarted dialysis. The decision to remove the graft was made on clinical grounds such as recurrent infections, pain or to make a future transplantation procedure anatomically possible. Male patients comprised 61% of the CAN group and the mean time from transplantation to recommencing dialysis was 56.4 months (range 3.6 to 169 months). The mean time from recommencing dialysis to undergoing a graft nephrectomy was 6.4 months (range 0.3 to 28 months) whilst 34.5% of patients had experienced a previous episode of acute rejection (table 3-1). Control tissue was obtained from macroscopically normal areas of nephrectomy specimens removed for renal carcinoma (n=19). The mean age of these patients was 53 years old at the time of nephrectomy

Table 3- 1 Details of patients with CAN whose renal tissue was studied in this work

Number of patients	28 (29 discrete nephrectomies)
Sex	F:11 39% M:17 61%
Age at transplant	14-74yrs mean 36.7
Original pathology PCKD Reflux nephropathy IgA nephropathy Diabetic nephropathy GN hereditary Membranous FSGS Alports Renal dysplasia Congenital obstructive uropathy Atheromatous renovascular disease Unknown	5 4 2 4 1 1 3 3 1 1 1 3
Blood groups O A B AB Unknown	12 8 5 1 3
Dialysis pre transplant: HD CAPD Unknown	21 6 2
Time on dialysis – pre transplantation (days)	3-4302 mean 717
Date of transplant	1986-2004
Donor: Cadaveric Living	22 7
Donor age (years)	18-70 mean 46.7
Mismatch	Full house 0 mismatch in 4 14%
Acute rejection episodes 0 1 2 unknown	13 9 1 6
time from transplantation to recommencing dialysis (months)	3.6 – 169 mean 56.4
Time from recommencing dialysis to graft nephrectomy (months)	0.3-28 mean 6.4

3.3 Confirmation of endothelial staining in human tissue.

Further immunostaining using CD34, an alternative specific endothelial cell marker was performed and this demonstrated a very similar staining pattern to CD31 (fig 3-1)

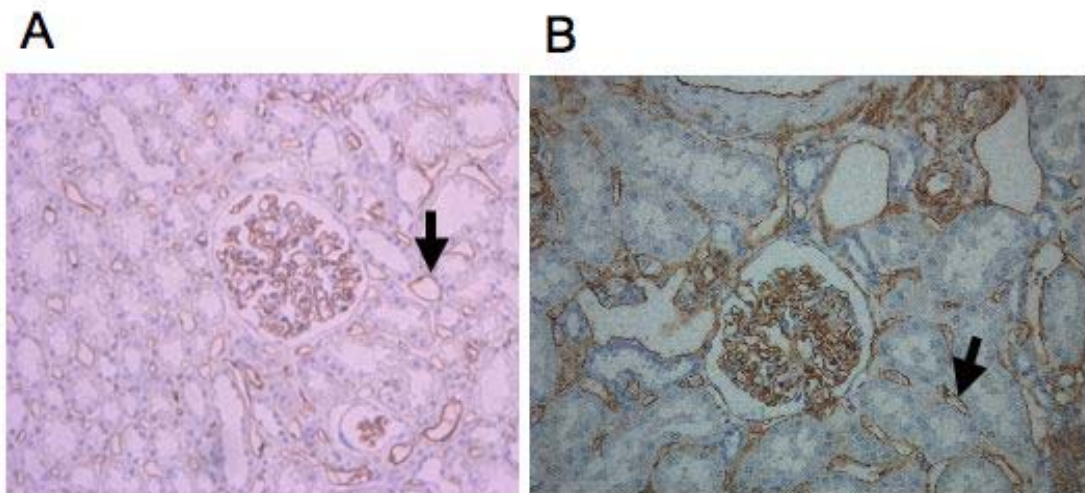


Figure 3- 1 Immunohistochemical staining of endothelial cells in human renal control tissue.

Peritubular capillaries in human renal tissue were identified by immunohistochemical staining using the endothelial cell markers CD31 and CD34

A Mouse anti human CD31 endothelial stain (example arrowed) (x200 magnification)

B Mouse anti human CD34 endothelial stain (example arrowed) (x200 magnification)

3.4 Upregulation of interstitial lymphatics is seen within CAN tissue.

In control kidney tissue the lymphatic vessels were located adjacent to arteries, as previously described with no lymphatic vessels evident in the interstitium (fig 3-2A). Immunostaining for lymphatic vessels was specific as they were identified with three different lymphatic markers: podoplanin (2 separate antibodies to podoplanin), LYVE-1 and VEGFR-3 (fig 3-2 C&D). In the CAN tissue, 17 assessable cases showed podoplanin positive lymphatic vessels within the interstitium not associated with arteries but in close proximity to glomeruli and tubules (fig 3-2B) (some tissue sections did not show any podoplanin positivity and were therefore excluded). In addition, double immunostaining for CD68 and podoplanin demonstrated several interstitial lymphatic vessels containing CD68 positive cells thereby implying that they may function to facilitate the exit of inflammatory cells from the kidney (fig 3-3).

Lymphatic vessels adjacent to large arteries or lying within the interstitium were quantified separately. Control and CAN tissue exhibited comparable numbers of lymphatic vessels situated in a perivascular location (fig 3-4). In contrast, interstitial lymphatic vessels were not identified in any control tissue section but significant numbers were evident within the diseased tubulointerstitium of grafts affected by CAN (fig 3-4).

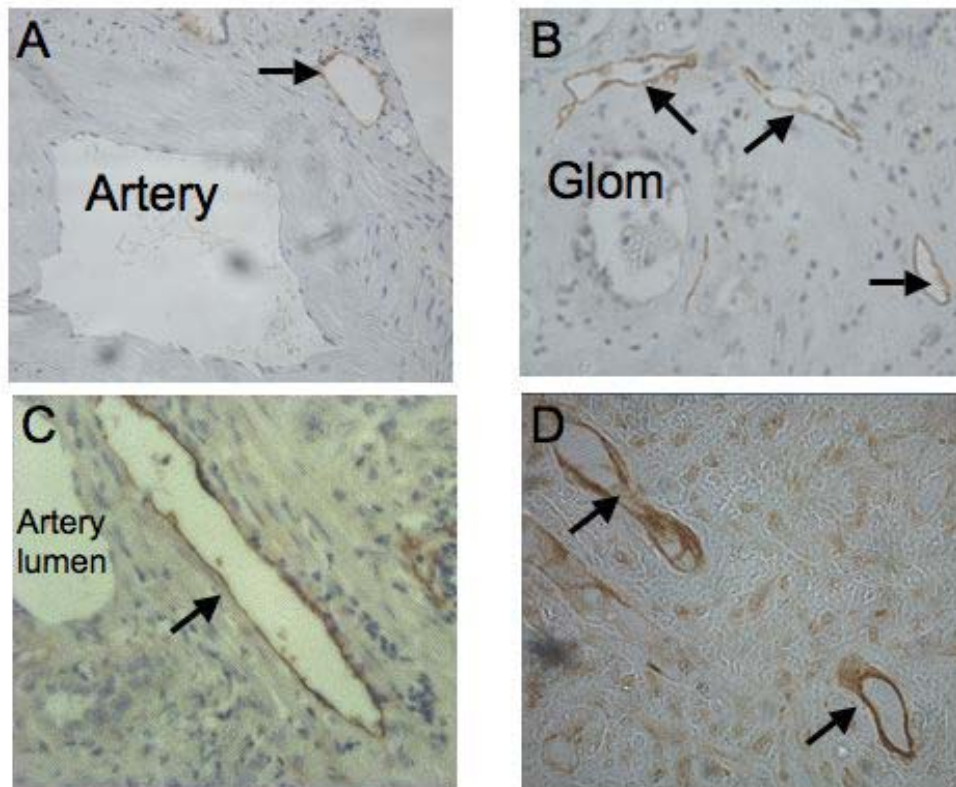


Figure 3- 2 Staining of lymphatic endothelium in human renal tissue

Lymphatic vessels in CAN and control tissue were identified by immunohistochemical staining for markers that discriminate vascular from lymphatic endothelial cells.

A) Immunostaining for podoplanin using the antibody D240 in control kidney tissue: Podoplanin positive lymphatic vessels (arrowed) are evident lying in close proximity to a large intra renal artery.

B) Immunostaining for podoplanin using the antibody D240 in CAN tissue; several podoplanin positive lymphatic vessels (arrowed) are evident within the tubulointerstitium adjacent to an atrophic glomerulus with periglomerular fibrosis.

C) LYVE-1 immunostaining of a lymphatic vessel in control kidney tissue lying in close proximity to a large intra renal artery

D) VEGFR-3 immunostaining of a lymphatic vessel in CAN kidney tissue within the tubulointerstitium.

(x200 magnification)



Figure 3- 3 Lymphatic vessel containing CD68 +ve M ϕ in CAN tissue

Immunostaining of human CAN tissue. Double labelling with the lymphatic endothelial cell marker podoplanin (purple) (arrowed), and the M ϕ marker CD68 (brown). M ϕ are evident within the lumen of the lymphatic vessels suggesting that they are functional. (x400 magnification)

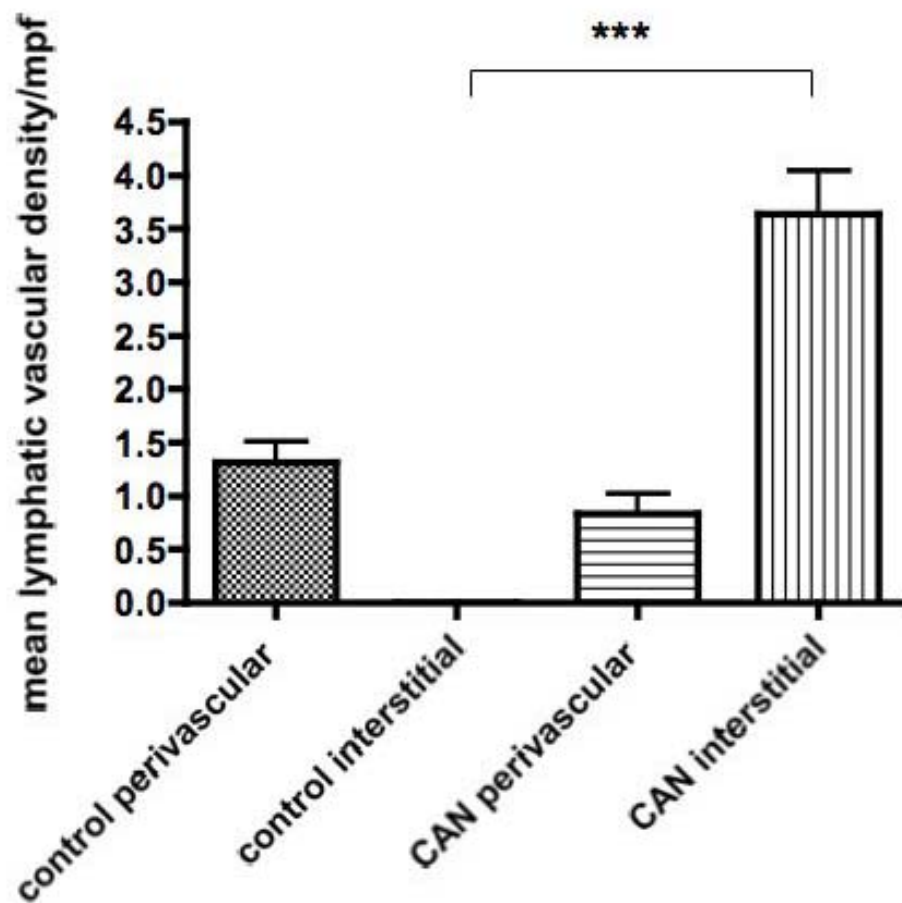


Figure 3- 4 Lymphatic vessels are found in the interstitium of human renal tissue in CAN

Lymphatic vessels in the perivascular and interstitial location were counted separately in tissue sections stained for podoplanin. In control kidney tissue, lymphatics were detected in the perivascular regions only. CAN tissue exhibited comparable numbers of perivascular lymphatic vessels but a marked increase in the number of lymphatic vessels was evident in the interstitium $p < 0.001^{***}$ (mpf = x200). (CAN N=17; control N=19)

3.5 Increased mononuclear cell infiltrate in CAN

Phenotyping the mononuclear cell infiltrate in CAN tissue revealed a mixture of M ϕ , T cells and B cells (fig 3-5). The B cell infiltrate was evident as both diffuse cell infiltration and as dense cellular aggregates. Control tissue exhibited negligible numbers of both B and T cells and as expected, low numbers of M ϕ . Semiquantitative scoring revealed a significant increase in the number of infiltrating interstitial CD68 positive M ϕ in both the cortex and the medulla in CAN tissue compared to controls (fig 3-6).

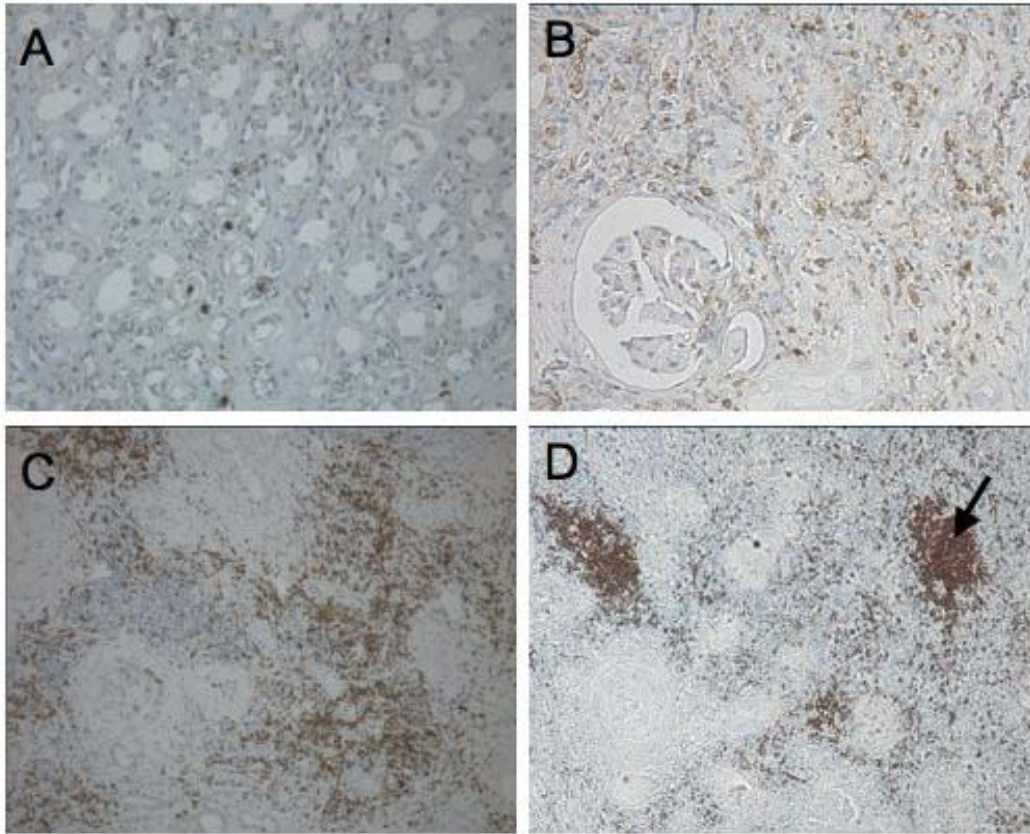


Figure 3- 5 Interstitial infiltrate of M ϕ , T cells and B cells are present in the CAN tissue.

Mononuclear cell infiltrate in CAN tissue was examined by immunostaining for leukocyte markers CD68 (M ϕ), CD3 (T cells) and CD20 (B cells).

M ϕ infiltration in control tissue **(A)** and CAN tissue **(B)**: Scattered CD68 positive M ϕ are present in control tissue with increased numbers evident in CAN tissue.

(C) Significant numbers of CD3 positive T cells are present within CAN tissue.

(D) CD20 positive B cells are scattered throughout the interstitium as well as forming dense aggregates (example arrowed)

(x200 magnification)

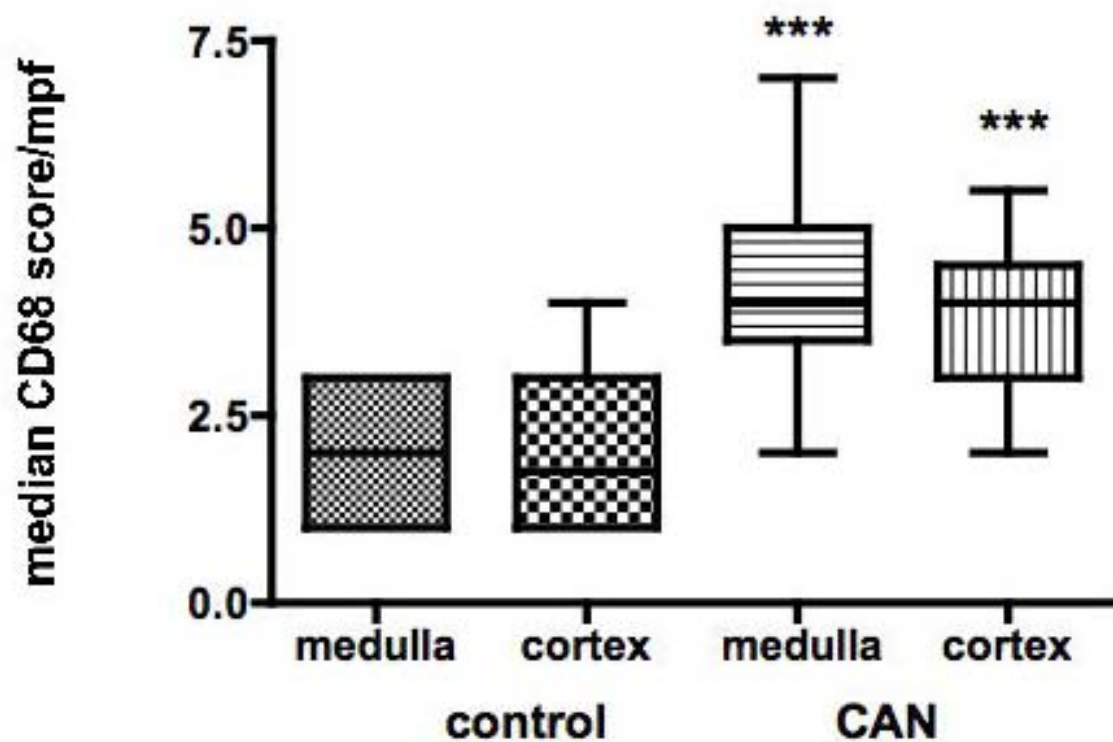


Figure 3- 6 Increase in interstitial M ϕ infiltrate is seen in the CAN tissue compared to control kidney tissue

Semi quantitative scoring of CD68 positive M ϕ infiltration indicated a significant increase in cortical and medullary infiltration of CAN tissue compared to controls $p < 0.001$ ***

3.6 iNOS expression seen in CAN tissue co-localised to macrophage infiltrate.

iNOS expression found in the interstitium of the CAN tissue was not visible in the interstitium of control tissue. In addition, dual immunofluorescent staining for CD68 and iNOS was performed and revealed a population of iNOS positive M ϕ within the interstitium of CAN grafts (fig 3-7).

3.7 Localisation of VEGF-A and -C expression

VEGF-C expression found in the interstitium of the CAN tissue was not apparent in the control tissue (fig 3-8). Non quantitative assessment of VEGF-A immunostaining suggested that expression was localised to podocytes and tubular epithelial cells in control tissue with preserved expression in CAN (fig 3-9). Dual immunofluorescent staining for the leukocyte markers CD68, CD3 and CD20 and VEGF-A and VEGF-C were performed. Occasional CD68 positive VEGF-C expressing cells were present within the interstitium of CAN tissue but neither B cells nor T cells expressed VEGF-C (fig 3-10). Interestingly, double labelling studies indicated striking co localisation of VEGF-A to interstitial B cell infiltrates whilst T cells and interstitial M ϕ did not express VEGF-A (fig 3-11).

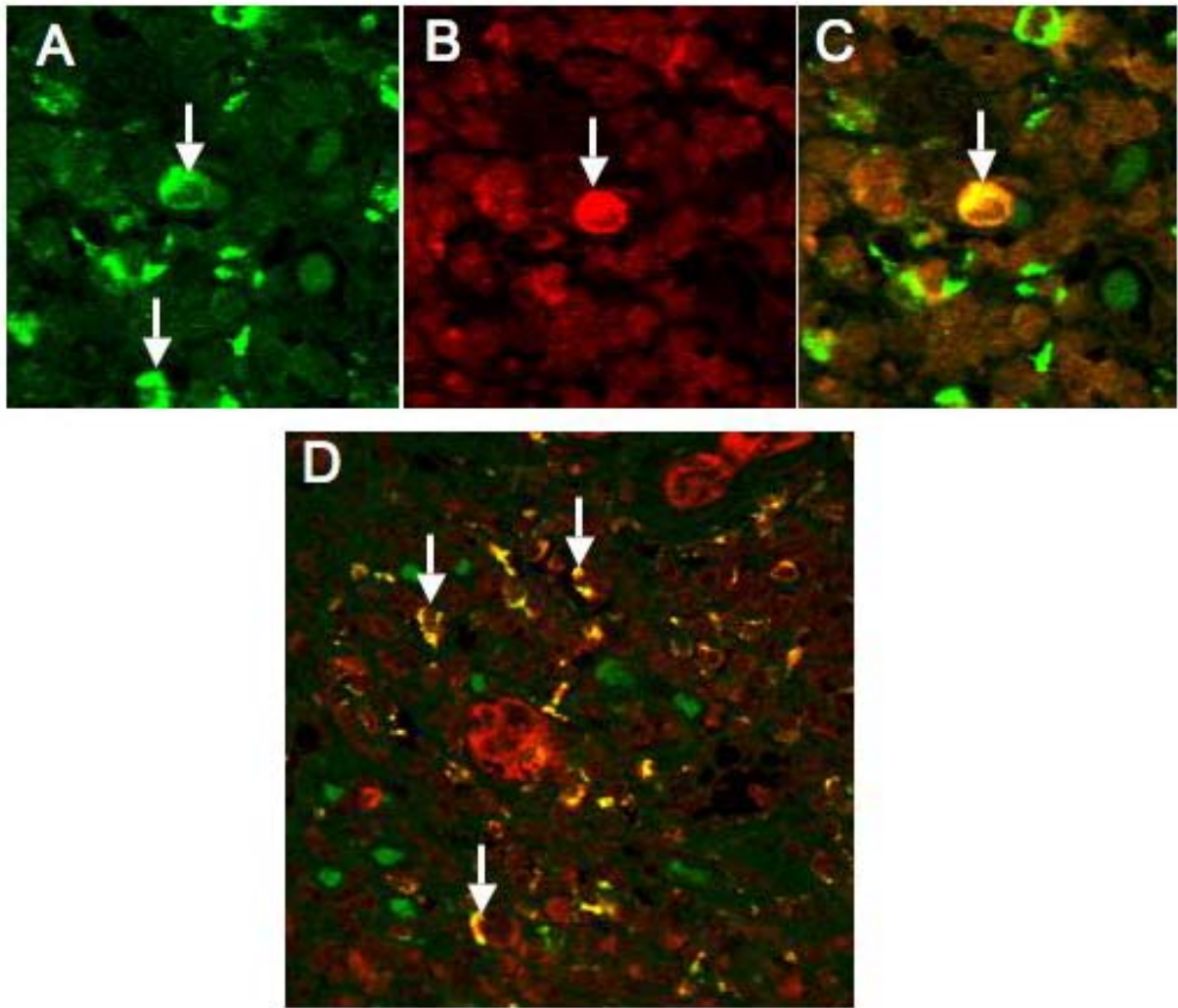


Figure 3- 7 Interstitial CD68 positive M ϕ express iNOS

Immunofluorescent staining of CAN tissue for the M ϕ marker CD68 (green) and iNOS (red).

A CD68 positive M ϕ are evident within the interstitium (example arrowed).

B Expression of iNOS is evident in tubular epithelial cells as well as within the interstitium (example arrowed)

C The merged image demonstrates co localisation of CD68 and iNOS indicating an iNOS positive M ϕ (example arrowed) (x400 magnification).

D A low power view of the merged CD68/iNOS image showing multiple CD68 positive cells expressing iNOS (example arrowed) (x200 magnification).

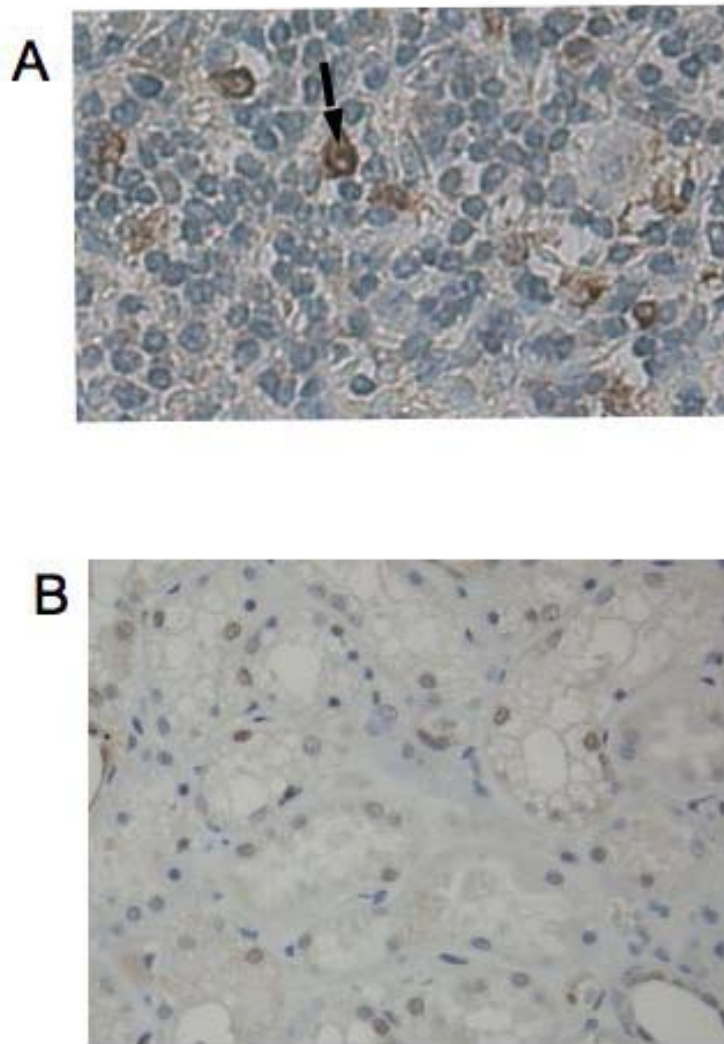


Figure 3- 8 VEGF-C immunostaining of human kidney tissue
A VEGF-C expression present in the interstitium of the CAN tissue (arrowed)
B VEGF-C expression is absent in control tissue (x200 magnification)

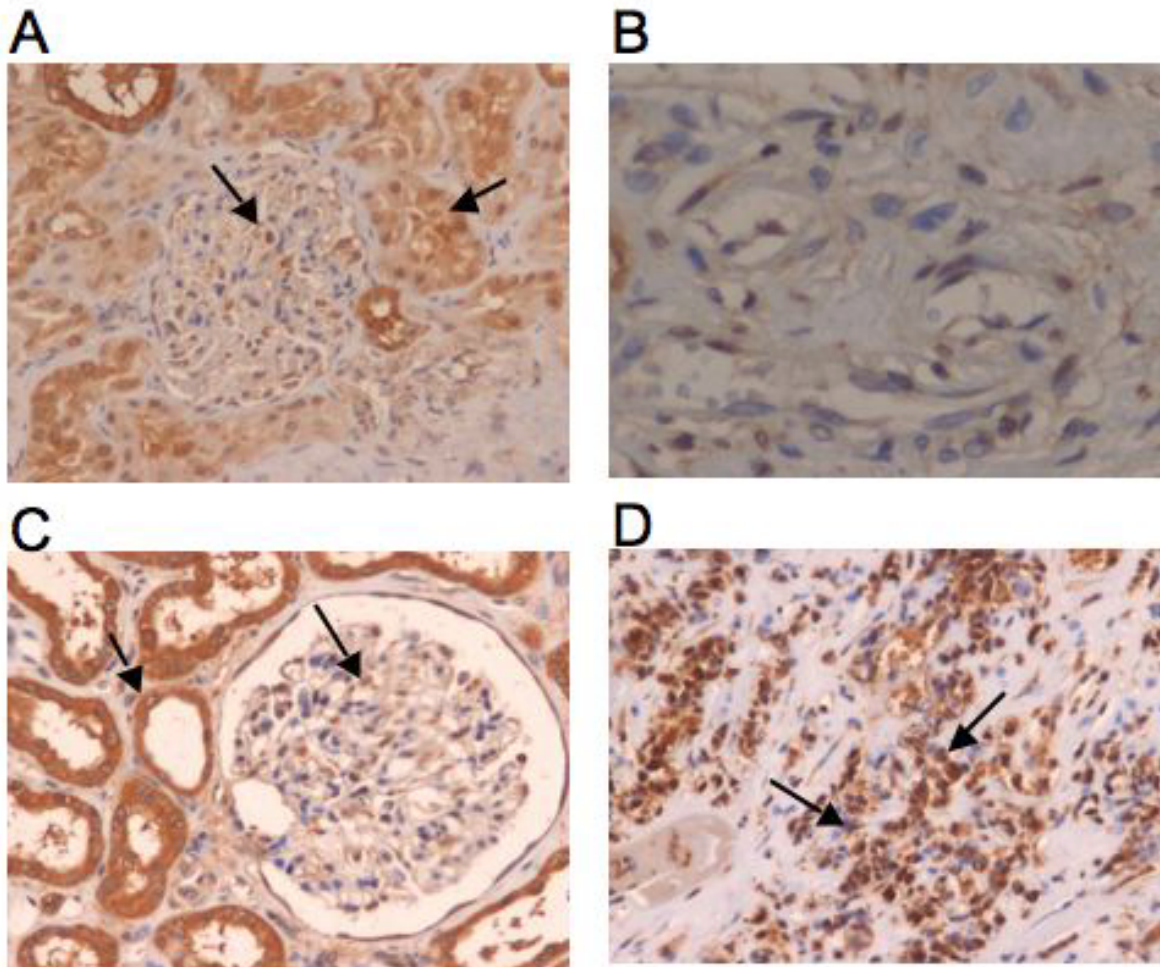


Figure 3- 9 Immunohistochemical staining of VEGF-A on human kidney tissue

In control tissue VEGF-A expression is localised to the podocytes and tubular epithelial cells (arrowed) **(A)** with very low expression seen in the interstitium **(B)**. In CAN tissue this podocyte and tubular epithelial expression is preserved **(C)** but significant interstitial VEGF-A expression is now also evident **(D)** (x200 magnification)

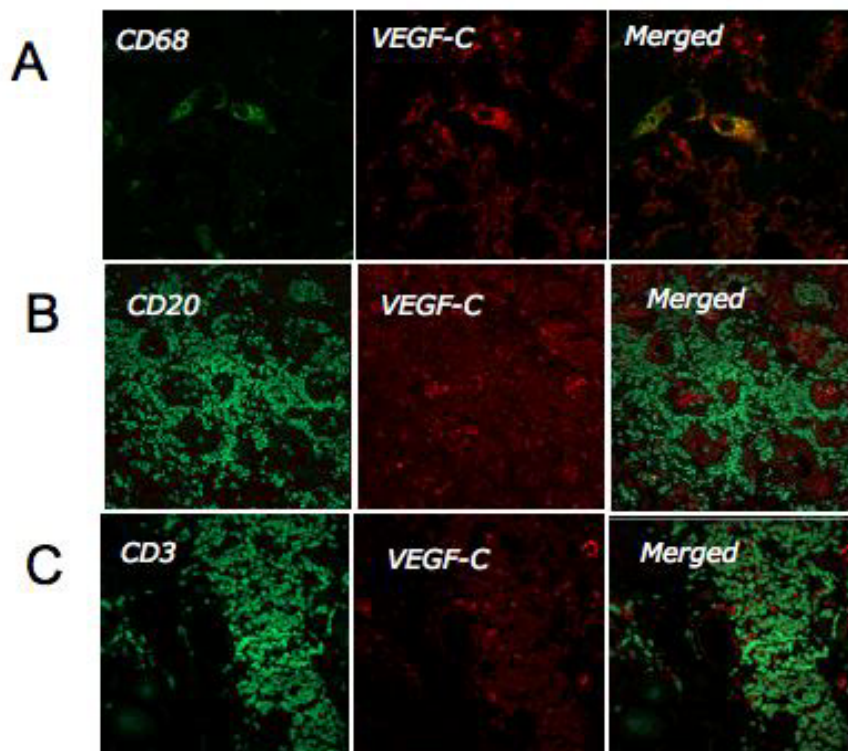


Figure 3- 10 VEGF-C expression by the inflammatory infiltrate in CAN tissue.

Dual immunofluorescent staining of CAN tissue was performed for the leukocyte markers CD68, CD3 and CD20 and VEGF-C.

A Co-localisation of CD68 (green) and VEGF-C (red) demonstrates M ϕ expression of VEGF-C

B CD20 (green) and VEGF-C (red) do not co localise showing that B cells are not expression VEGF-C.

C CD3 (green) and VEGF-C (red) do not co localise showing that T cells are not expressing VEGF-C
(x200 magnification)

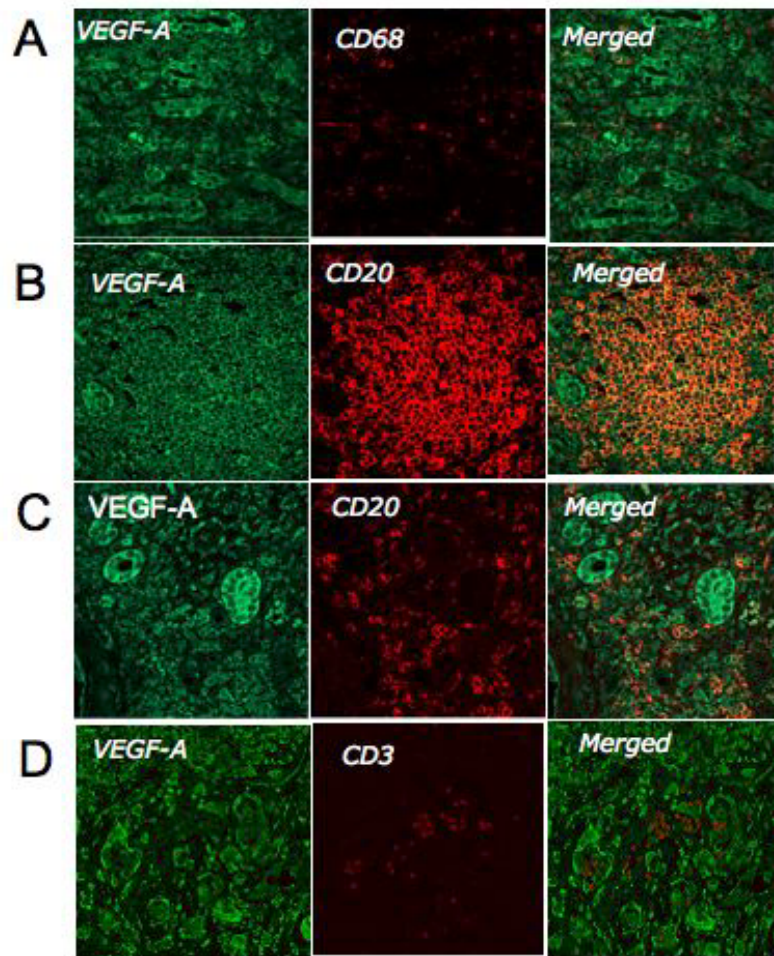


Figure 3- 11 VEGF-A expression by the inflammatory infiltrate in CAN tissue.

Dual immunofluorescent staining of CAN tissue was performed for the leukocyte markers CD68, CD3 and CD20 and VEGF-A.

A VEGF-A (green and CD68 (red) do not co localise showing that M ϕ are not expressing VEGF-A

B Striking co-localisation of CD20 (red) and VEGF-A (green) demonstrates aggregate B cell expression of VEGF-A

C Striking co localisation of CD20 (red) and VEGF-A (green) demonstrates dispersed B cells also express VEGF-A

D CD3 (red) and VEGF-A (green do not co localise showing that T cells are not expressing VEGF-A
(x200 magnification)

3.8 Analysis of nephrectomy grafts with continued immunosuppression show comparable changes to results seen including all grafts

In order to determine whether differences existed between those patients who had continued immunosuppression compared to those whose immunosuppression had been withdrawn analyses between these two groups was carried out. In the patients whose immunosuppression has been stopped (n=19) the renal grafts may suffer acute rejection. In order to determine that the changes seen in the grafts are due to CAN and not acute rejection the sections from patients whose immunosuppression was continued at the time of nephrectomy were analysed separately (n=10).

In this subset of tissue results were comparable to those reported from the group who had stopped immunosuppression except that there was a significant increase in cortical M ϕ infiltrate in those continuing immunosuppression compared to those whose immunosuppression had been stopped (table 3-2).

Table 3- 2 Comparison between patients with immunosuppression continued or discontinued

	Group A Immunosuppression discontinued	Group B Immunosuppression maintained	Group C Control group
Cortical CD68 counts	3.68±0.18	4.7±0.15 (p<0.001 vs Gp A)	1.86±0.23 (p<0.001 vs Gp A and Gp B)
Medullary CD68 counts	4.05±0.22	4.4±0.18 (p=NS vs Gp A)	1.92±0.19 (p<0.001 vs Gp A and Gp B)
Cortical Chalkley counts (CD31)	5.13±0.16	4.8±0.33 (p=NS vs GpA)	22.45±45 (p<0.001 vs Gp A and Gp B)
Medullary Chalkley counts (CD31)	5±0.21	5.2±0.32 (p=NS vs Gp A)	21.48±0.56 (p<0.001 vs Gp A and Gp B)
Perivascular lymphatic vessel number	0.66±0.18	1.08±0.38 (p=NS vs Gp A)	1.31±0.39 (p=NS vs Gp A or Gp B)
Interstitial lymphatic vessel number	3.15±0.46	4.34±0.67 (p=NS vs Gp A)	

3.9 Summary & discussion

In summary the studies outlined above demonstrate the following findings:

1. CAN tissue exhibited an increase in number of lymphatic vessels within the interstitium.
2. An increased interstitial M ϕ infiltrate was evident in both the medulla and cortex of CAN tissue compared to the control group.
3. Interstitial infiltrate of both B and T cells was present in the CAN tissue but not present in the control group. The B cell infiltrate was both nodular and diffuse.
4. Interstitial iNOS and VEGF-C expression is seen in the CAN tissue but not control tissue.
5. VEGF-A expression is seen in the tubules of the control tissue. This is preserved in the allograft with the addition of interstitial VEGF-A expression.
6. Some cells within the M ϕ infiltrate appear to express both iNOS and VEGF-C but not VEGF-A.
7. The B cells express VEGF-A but not VEGF-C and the T cells express neither of these growth factors.

The human tissues used in these studies do have their limitations.

The CAN tissues examined came from patients who are back on dialysis with the majority of patients off their immunosuppressants.

However by analysing the patients whose immunosuppression had not been discontinued separately to those whose treatment had been withdrawn we found no difference between the groups (except an increase cortical CD68 M ϕ in the immunosuppressed group) suggesting that our findings were related to CAN and were not simply due to stopping immunosuppression.

These patients have ESRF and therefore we are seeing the process at a late stage. Ideally we would have also studied earlier biopsy tissue from these grafts as well as biopsy tissue from functioning grafts at a similar time post transplantation. This would have provided information about the progression of microvascular loss and lymphangiogenesis under normal circumstances. Unfortunately protocol biopsies are not carried out routinely at our centre and the tissue available from previous diagnostic biopsies was limited and not available for research purposes.

The mean age of the patients from which the control renal tissue was taken was higher than that in the CAN group suggesting that age is not a confounding factor in this process and that the changes seen in microvascular density are not age related but in fact are due to CAN.

The ideal control tissue would have been allograft tissue from a functioning graft with no evidence of acute or chronic rejection.

Fixation of the tissues used in the studies was variable and some may have been left in formalin for several hours/days before being prepared. It is important to remember that CD31 is a pan endothelial cell marker and therefore does not distinguish between lymphatic and vascular endothelium. PAL-E is a specific VEC marker but is only effective on frozen sections, which was not available for these human tissue studies. We did however carry out a CD34 stain, which produced the same pattern of staining as our CD31 stain and confirmed that we were in fact staining endothelium rather than some other cell type.

In figure 3-4 looking at podoplanin expression in the CAN vs. Control we see both an up regulation of lymphatics in the interstitium and a slight though not significant reduction in the perivascular number of lymphatic vessels. In the CAN tissue there is a loss of kidney tissue due to the disease, which could explain the apparent reduction of lymphatic number (but not significant) in the normal location.

Analysis of proliferation in these lymphatics was not possible as multiple attempts at double staining using the proliferation marker ki67 and podoplanin or D240 were unsuccessful.

Chapter 4.

The role of nitric oxide in M ϕ mediated endothelial cell apoptosis

4.1 Introduction

Previous studies have demonstrated that M ϕ activated with LPS and INF γ induce direct apoptosis of both mesangial and primary tubular epithelial cells (Duffield et al., 2000; Kipari et al., 2006). In these systems NO appeared to act as the key death effector supporting earlier work showing a correlation between iNOS expression, M ϕ infiltration and apoptosis (Szabolcs et al., 1996) .

This thesis tested the hypothesis that activated M ϕ induce direct endothelial cell death through the production of the death effector NO. In the studies outlined in this chapter a well established microscopically quantifiable *in vitro* co-culture assay was used. (Duffield et al., 2000; Kipari et al., 2006) to study the cytotoxic interaction between BMDM and MCEC-1 cells. This chapter describes the results of these studies that compared four experimental conditions.

- MCEC-1 cells alone (control group)
- MCEC-1 cells activated with the cytokines INF γ and LPS
- Non-activated co-cultures of MCEC-1 cells and BMDM
- Co-cultures activated with INF γ and LPS

Each experiment was repeated at least 3 times and each time the condition was carried out in triplicate. 5 fields in each well were examined and the number of apoptotic cells, mitotic cells and the total number MCEC-1 cells present were counted. Data was expressed as a mean \pm SEM per high power field.

4.2 Co-cultures exhibit reduced endothelial cell mitosis that is independent of cytokine stimulation.

Proliferation of endothelial cells was identified by the presence of mitotic cells seen by fluorescent microscopy (fig 4-1).

The first observation was that mitosis was evident in control non-activated MCEC-1 cell cultures and this was not affected by cytokine activation (fig 4-2 A). However this endothelial cell proliferation was completely inhibited when MCEC-1 cells were co-cultured with activated M ϕ . In addition, proliferation was markedly inhibited when the MCEC-1 were co-cultured with non activated M ϕ indicating that M ϕ inhibition of endothelial cell proliferation is independent of cytokine activation (fig 4-2 A).

Time course studies showed that at 6 hours in co-culture with activated M ϕ endothelial cell proliferation was reduced by 76% with complete inhibition evident at 12hours (fig 4-2 B)

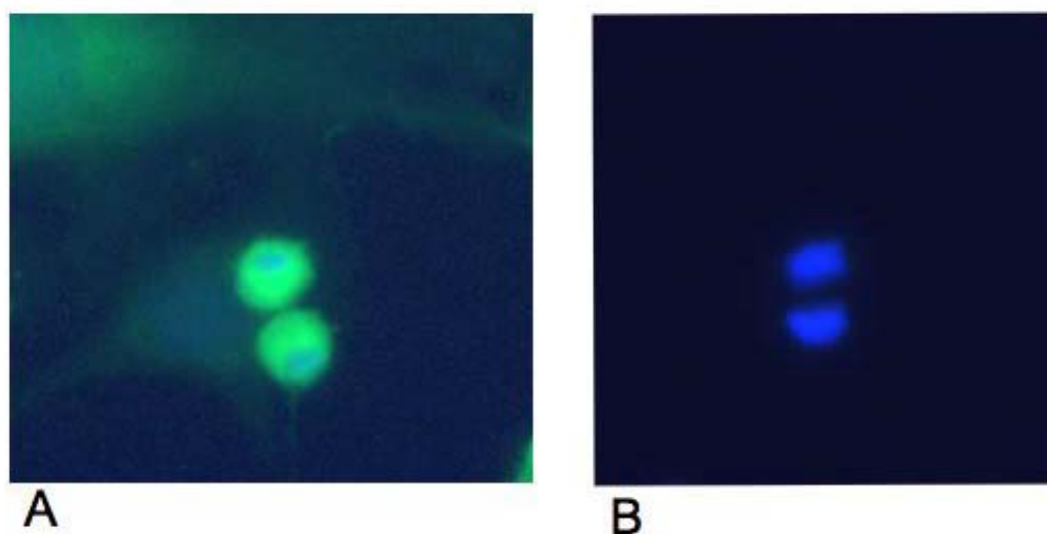


Figure 4- 1 Proliferating endothelial cells were evident in control co-culture wells.

Cell tracker green labelled MCEC-1 cells were cultured with non-activated M ϕ for 24hours at 37⁰C. Following fixation with formaldehyde and counterstain with Hoechst 33342 analysis was carried out using fluorescent microscopy. Mitotic cells were identified by their characteristic chromatin pattern.

A CM green labelled endothelial cell undergoing mitosis

B Mitotic cells highlighted by Hoechst staining.

(High power view x320 magnification)

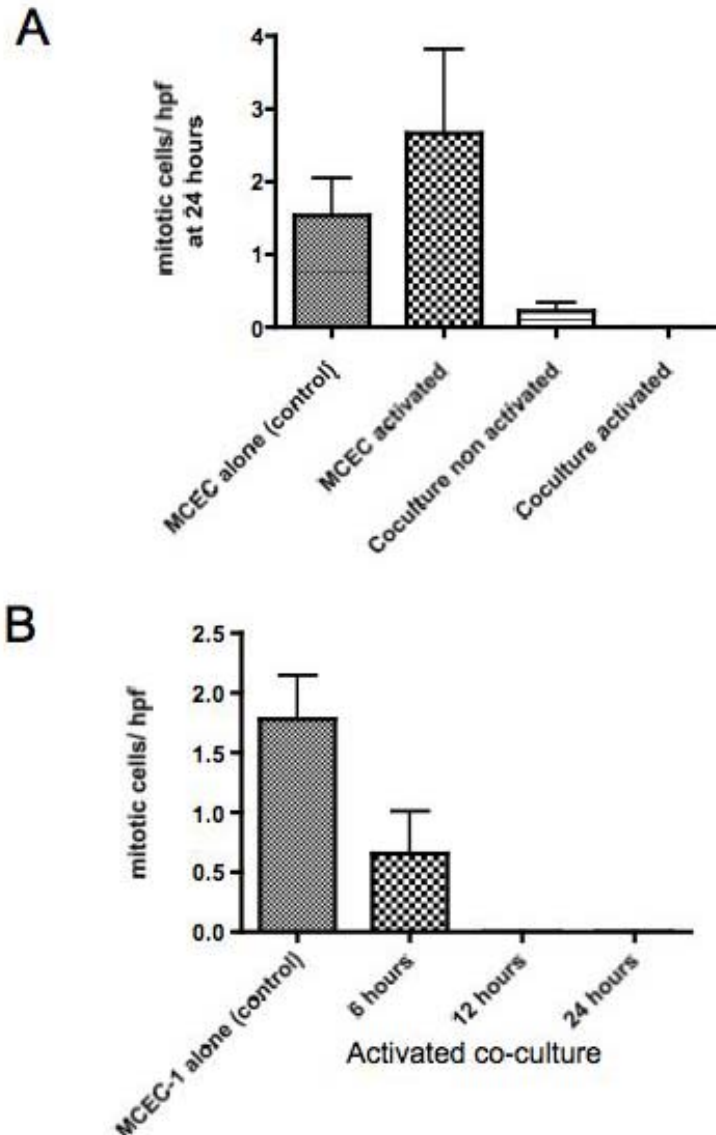


Figure 4- 2 Inhibition of endothelial cell proliferation is M ϕ dependant but cytokine independent.

Cell tracker green labelled MCEC-1 were cultured alone or in the presence of cell tracker-orange labelled BMDM (M ϕ :MCEC 2:1 ratio) with or without LPS (1 μ g/ml) and IFN- γ (100u/ml). After incubation at 37 $^{\circ}$ C co-cultures were fixed with formaldehyde, counterstained with Hoechst 33342 and analysed using fluorescent microscopy (n=3)

A Expressing mitosis as the number of mitotic cells per high power field. After 24 hours incubation proliferation is completely abolished in activated co-cultures and markedly inhibited in non-activated co-cultures. There was no significant difference in the level of proliferation between activated and non-activated groups.

B Time course studies indicated inhibition of endothelial cell proliferation in activated co-cultures was present from 6 hours

4.3 Cytokine activated co-cultures exhibit increased endothelial cell apoptosis

Apoptotic endothelial cells were identified by their condensed cytoplasm and pyknotic nucleus seen using fluorescent microscopy. (fig 4-3). The number of apoptotic cells were counted and expressed as the number of apoptotic cells per high power field.

The first thing observed was that no apoptosis was seen in endothelial cells cultured alone or in the presence of the activating cytokine IFN- γ and LPS proving that addition of cytokines alone was not cytotoxic to MCEC-1 cells. However the activated co-culture at 24 hours exhibited a significant level of apoptosis that was significantly higher to that seen in non activated co-cultures at 24 hours thus indicating that induction of MCEC-1 cell apoptosis was dependent upon cytokine activation of M ϕ (fig 4-4 A).

Kinetic analysis demonstrated that in the activated co-cultures there was no apoptosis apparent at 3 hours but increasing levels were seen from 6 hours (fig 4-4B).

A low level of phagocytosis was seen in activated co-cultures at 12 and 24 hours, but this was not quantified as it was an infrequent finding (fig 4-5).

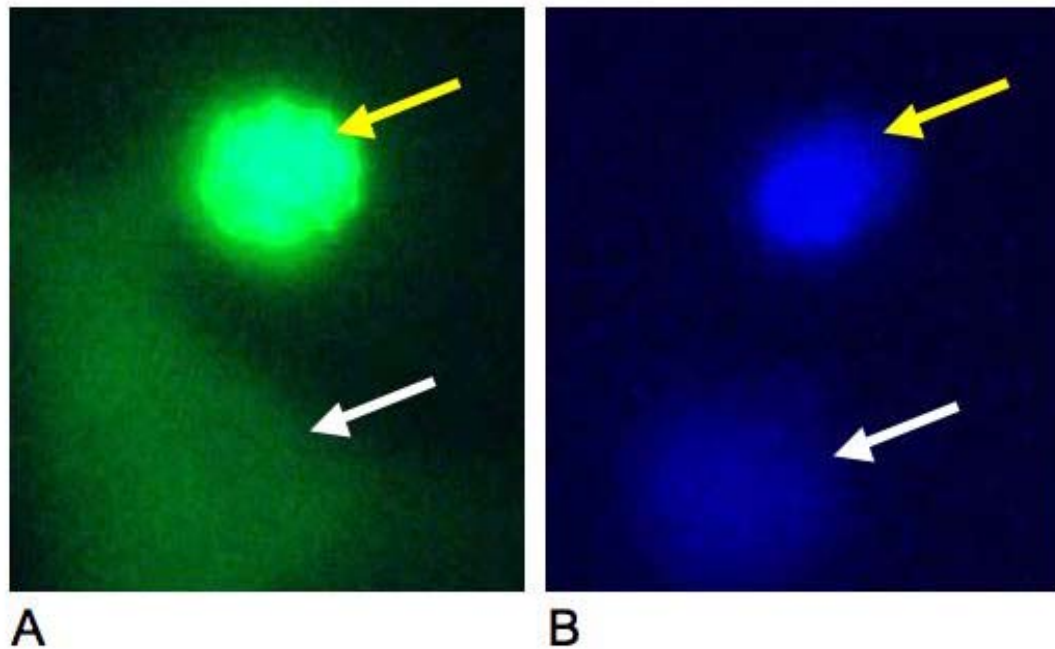


Figure 4- 3 Apoptotic endothelial cells in activated co-cultures

Cell tracker-green labelled MCEC-1 cells were cultured in the presence of mature cell-tracker orange labelled BMDM. Selected co-cultures were activated with LPS/INF- γ . Following incubation for 24 hours at 37°C the cells were fixed with formaldehyde and counter stained with Hoechst 33342. Using fluorescent microscopy apoptotic MCEC-1 cells were identified by their condensed cytoplasm and pyknotic nucleus.

A CM green labelled apoptotic MCEC-1 cell (yellow arrow). CM green labelled healthy MCEC-1 cell (white arrow).

B Hoechst stain demonstrating the pyknotic nucleus of the apoptotic cell (yellow arrow). Hoechst stain demonstrating the normal nucleus of the healthy MCEC-1 cell (white arrow)

(High power view x320 magnification)

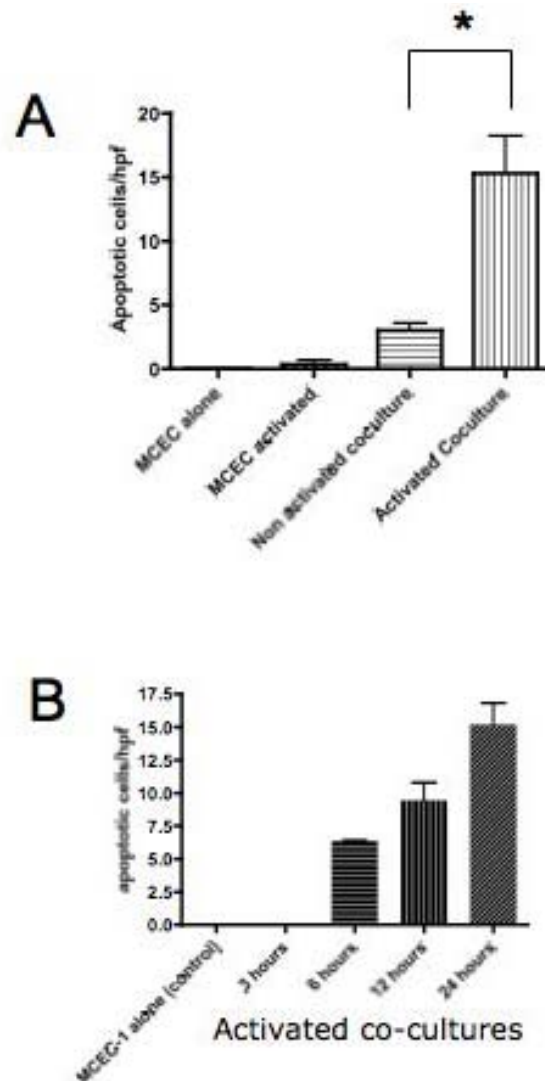


Figure 4- 4 Cytokine activated M ϕ induce MCEC-1 cell apoptosis.

Cell tracker green labelled MCEC-1 were cultured alone or in the presence of cell tracker-orange labelled BMDM (M ϕ :MCEC 2:1 ratio) with or without LPS (1mg/ml) and IFN- γ (100u/ml). After incubation at 37°C co-cultures were fixed with formaldehyde counterstained with Hoechst 33342 and analysed using fluorescent microscopy (n=3).

A Expressing apoptosis as the number of apoptotic cells per high power field. After 24 hours incubation cytokine activated M ϕ in co-culture induce significant MCEC death compared to non activated M ϕ $p < 0.05^*$

B A time course study was undertaken. MCEC-1 death appears to be induced by 6 hours in co-culture with activated M ϕ . There is no significant difference in the level of apoptosis between 6 and 12 hours or 12 and 24 hours

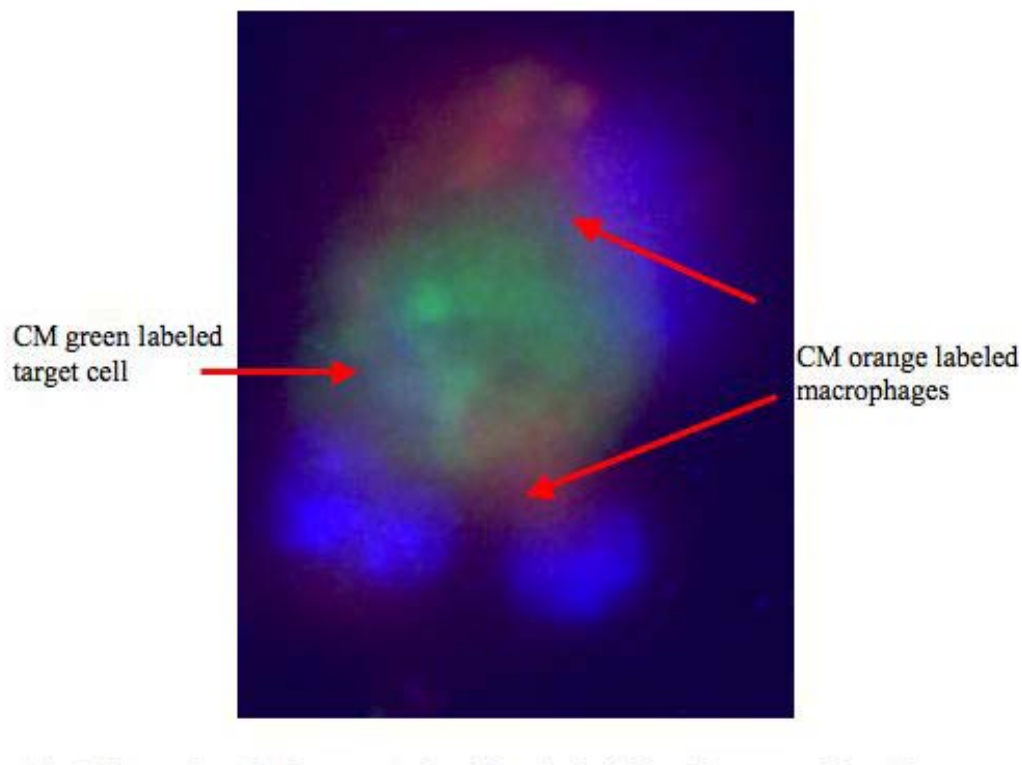


Figure 4- 5 Occasional phagocytosis of endothelial cells was evident in activated co-cultures.

Cell tracker green labelled MCEC-1 cells were cultured in the presence of mature cell-tracker orange labelled BMDM. Selected co-cultures were activated with LPS/INF- γ . Following incubation for 24 hours at 37°C the cells were fixed with formaldehyde and counterstained with Hoechst 33342. Using fluorescent microscopy occasional orange labelled M ϕ could be seen ingesting green MCEC-1 cells. (x320 magnification)

4.4 Cytokine activation of co-cultures results in a reduction of total number of MCEC-1 cells.

Total endothelial cell number per hpf was determined and expressed as a percentage of the number of endothelial cells in control wells (depicted as 100%).

The first finding was that culture of MCEC-1 cells with IFN- γ and LPS did not affect endothelial cell number indicating that IFN- γ and LPS are not inherently cytotoxic. Co-culture of MCEC-1 cells with non-activated BMDM for 24 hours did not induce a significant reduction in endothelial cell number. There was however a significant reduction in endothelial cell number in cytokine activated co-cultures compared to the control group (MCEC-1 cells alone) (fig 4-6A).

The second observation was that there was a significant reduction in MCEC-1 cell numbers seen in the activated co-cultures compared to the non activated co-culture indicating that cytokine activation is key (fig 4-6A).

Endothelial cell numbers were compared in the activated co-culture with the control group of MCEC-1 cells cultured alone at various time points. This indicated that a reduction in total MCEC-1 cell number in the activated co-cultures was only evident after 6 hours but this reduction only became significant at 12 hours (fig 4-6B).

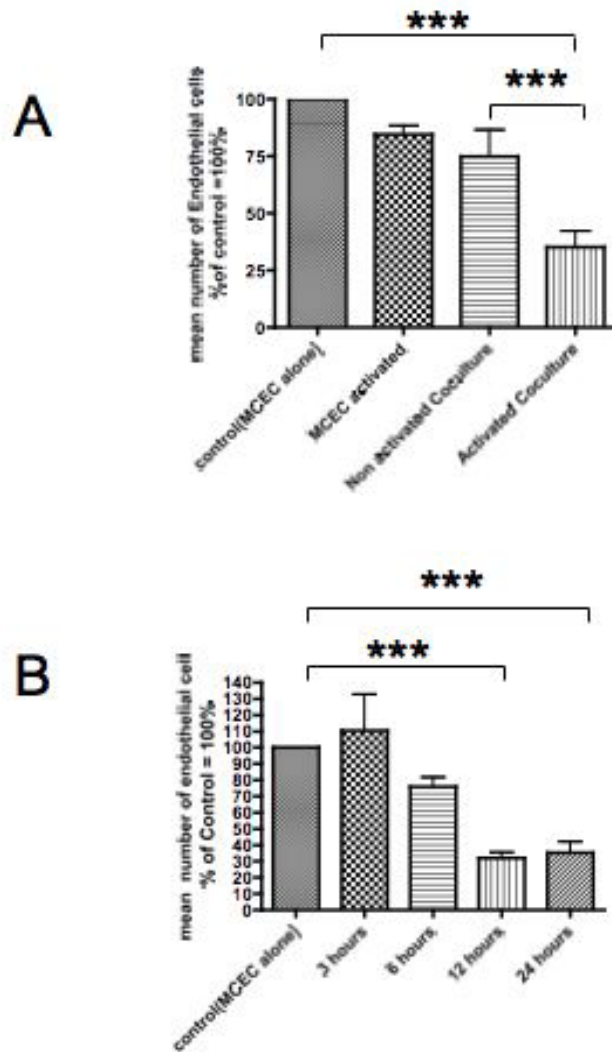


Figure 4- 6 Reduction in endothelial cell number is only seen in activated co-culture.

Cell tracker green labelled MCEC-1 cells were cultured alone or in the presence of cell tracker-orange labelled BMDM (M ϕ :MCEC 2:1ratio) with or without LPS (1 μ g/ml) and IFN- γ (100u/ml). After incubation at 37⁰C co-cultures were fixed with formaldehyde and counterstained with Hoechst 33342. MCEC-1 cell number was expressed as the percentage of control cell number, which was designated as 100%.

A There was a significant reduction in the MCEC-1 cell number expressed as a percentage of MCEC-1 cells in the activated co-cultures compared to both the control group and the non activated co-cultures $p < 0.001$ ***

B Kinetic analysis demonstrated MCEC-1 loss beginning at 6 hours ($p = 0.054$) increasing to significant loss by 12 hours and 24 hours $p < 0.001$ ***

4.5 Transferability of death signal

In order to determine whether soluble mediators present in the supernatant from activated BMDM could induce apoptosis of MCEC-1 cell experiments were performed involving transfer of supernatant from both non-activated and cytokine activated BMDM to MCEC-1 cells. Conditioned supernatant from cytokine activated BMDM did not induce endothelial cell loss suggesting that the death effector responsible is either short lived or membrane bound (fig 4-7). Preliminary studies suggested that there was no effect on either proliferation or apoptosis. This was consistent with the findings of earlier work carried out by colleagues when studying the effect of supernatant from cytokine activated BMDM on tubular epithelial cells. No effect on proliferation or apoptosis was found in this setting. There was therefore no precedent to formally repeat these experiments using the MCEC-1 cells.

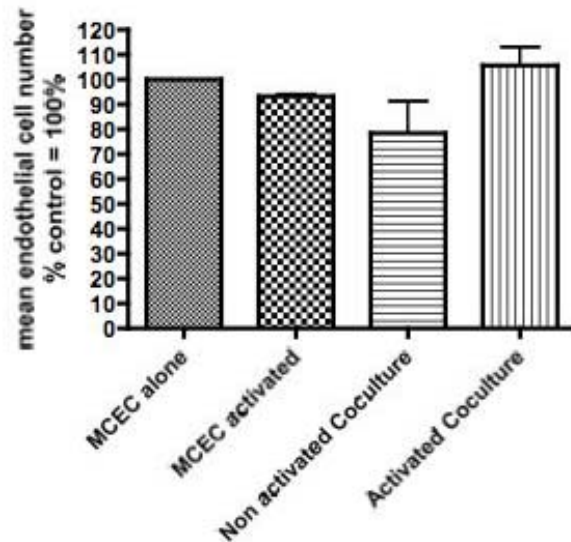


Figure 4- 7 The M ϕ death signal is not transferable.

Conditioned supernatant derived from 250,000 mature BMDM/well and cultured for 24 hours in the presence or absence of LPS (1 μ g/ml) and IFN- γ (100U/ml) was harvested and added to MCEC-1 (50,000 cells/well). Following 24 hours incubation with the M ϕ supernatant, the wells were fixed with formaldehyde and endothelial cell number assessed using fluorescent microscopy.

MCEC-1 cell numbers are comparable to the control groups (MCEC alone or activated with LPS and IFN- γ) whether incubated with activated or non-activated M ϕ supernatant.

This would suggest that the death effector is either short acting or membrane bound.

4.6 Video microscopy

Co-culture between IFN- γ and LPS activated M ϕ and MCEC-1 cells was examined using video microscopy over an 18 hour time period with pictures being taken every 5 minutes as described in chapter 2. Using this method the interaction between the M ϕ and MCEC-1 cell could be visually assessed. The M ϕ appeared to be very dynamic moving freely within this system and the MCEC-1 cells could be seen lying both separately and in direct contact to the activated M ϕ . Both the MCEC-1 cells in direct contact as well as those lying some distance from the M ϕ were observed undergoing apoptosis with condensation of their cytoplasm and cell shrinkage. This demonstrated MCEC-1 apoptosis that was both dependent and independent of direct contact with the activated M ϕ . (see enclosed DVD)

4.7 Mediators of apoptosis

4.7.1 Nitric oxide is implicated in this system

Previous work in our group indicated the involvement of NO in both mesangial cell and tubular epithelial cell apoptosis (Duffield et al., 2000; Kipari et al., 2006). In the following experiments L-NIL a pharmacological inhibitor of iNOS was included in co-culture experiments with MCEC-1 cells to determine whether M ϕ derived NO was also involved in the induction of apoptosis of MCEC-1 cells.

The generation of NO was determined by the Griess assay that measures nitrite, a stable breakdown product of NO. The Griess assay demonstrated that BMDM activated with LPS and IFN γ produce significant amounts of NO when compared to non-activated M ϕ (fig 4-8).

Cytokine activated BMDM were then cultured in the presence of L-Nil or its inactive isomer D-Nil. The addition of L-Nil at a concentration as low as 10 μ mol was able to inhibit this nitrite production. Maximal inhibition was seen to occur with the addition of 30 μ M of L-Nil with no significant reduction seen with addition of an equal concentration of D-Nil (fig 4-8). A concentration of 30 μ M was chosen for both L-Nil and D-Nil in future experiments.

Co-culture experiments were performed as previously described with the addition of L-Nil or D-Nil to the culture medium of the activated co-cultures. In the activated co-culture the number of apoptotic cells was 5 \pm 1.3 per hpf which was comparable to the level seen with the

addition of D-Nil (4.5 ± 0.5 per hpf). The addition of L-Nil significantly reduced the level of MCEC-1 cell apoptosis to 0.24 ± 0.14 per hpf (fig 4-9). The inclusion of L-Nil inhibited MCEC-1 cell apoptosis by $95 \pm 2.26\%$ suggesting that NO is a key component of M ϕ cytotoxicity in this assay.

As predicted the addition of L-Nil to the activated co-culture preserved MCEC-1 cell number with no significant difference in cell number compared to the control group. There was however a significant difference in total number of endothelial cells present in activated co-cultures with or without L-Nil (18.2 ± 1.7 vs. 10.2 ± 1.8 respectively; $p < 0.01$). D-Nil had no protective effect on MCEC-1 cell number (fig 4-10)

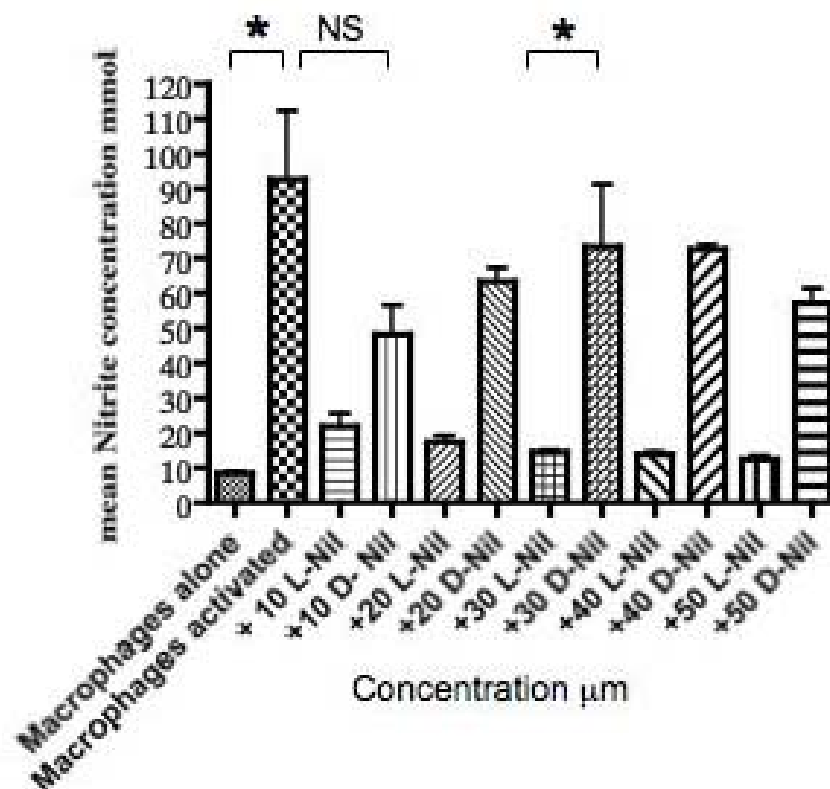


Figure 4- 8 Nitrite produced by activated M ϕ is effectively inhibited by L-Nil

Conditioned supernatant was derived from 250,000 mature BMDM/well cultured for 24 hours in the presence or absence of LPS (1 μ g/ml) and IFN- γ (100U/ml) with or without L-Nil or D-Nil (concentration ranging from 10 μ m-50 μ m). Griess assay was carried out on this supernatant to assess the concentration of nitrite produced from M ϕ under the above conditions.

There was significantly increased nitrite production from M ϕ activated with LPS and INF- γ compared to the non-activated M ϕ $p < 0.05^*$

Addition of L-Nil to the activated M ϕ inhibited nitrite production maximally with 30 μ mol of L-Nil not seen with the addition of its inactive isomer D-Nil $p < 0.05^*$

There was no significant difference in nitrite production between activated M ϕ alone or the addition of D-Nil

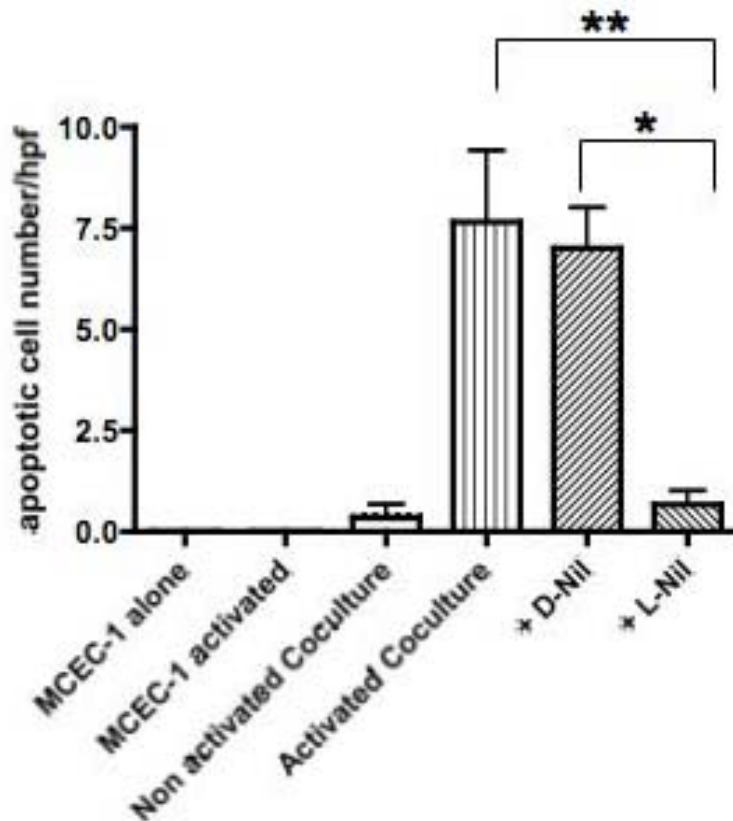


Figure 4- 9 Apoptosis was almost completely abolished with the addition of L-Nil but not D-nil.

Cell tracker green labelled MCEC-1 cells were cultured alone or in the presence of cell tracker-orange labelled BMDM. Cultures were activated with LPS (1 μ g/ml) and IFN- γ (100U/ml) in the presence or absence of the iNOS inhibitor L-Nil or its inactive isomer control D-Nil (final concentration 30 μ mol for both reagents). Cultures were incubated for 24 hours at 37 $^{\circ}$ C before fixation with formaldehyde and analysis using fluorescent microscopy.

There was a significant reduction in the number of apoptotic cells seen with L- Nil addition compared to both the activated co-culture or with the addition of D-Nil $p < 0.01^{**}$ and $p < 0.05^{*}$ respectively (n=3).

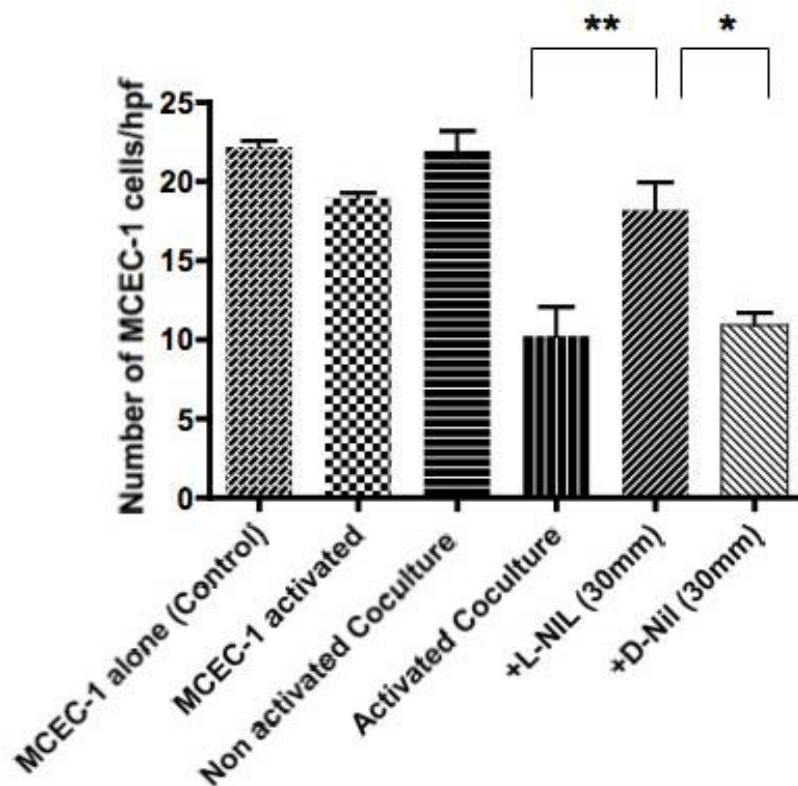


Figure 4- 10 Endothelial cell number is preserved by addition of L-Nil to activated co-cultures.

Cell tracker green labelled MCEC-1 were cultured alone or in the presence of cell tracker-orange labelled BMDM. Cultures were activated with LPS (1 μ g/ml) and INF- γ (100U/ml) in the presence of absence of the iNOS inhibitor L-Nil or its inactive isomer control D-Nil (final concentration 30 μ mol for both reagents). Cultures were incubated for 24 hours at 37°C before fixation with formaldehyde and analysis using fluorescent microscopy.

With the addition of 30 μ mol L-Nil there was no significant difference in endothelial cell number compared to the control group. There is a significant difference in endothelial cell number in the L-Nil group compared to either the activated co-culture or D-Nil groups $p < 0.01^{**}$ and $p < 0.05^{*}$ respectively (n=3)

4.7.2 TNF α production by activated macrophages is not inhibited by the addition of L-Nil

TNF α may also be an important death effector in this system. Thus a TNF α ELISA was performed to establish whether INF- γ and LPS activated M ϕ produce significant levels of TNF α and whether TNF α production was affected by the addition of L-Nil. Significant TNF α production by activated M ϕ was evident compared to non-activated M ϕ (2542 \pm 22 vs. 3.24 \pm 89pg/ml; $p < 0.001$). TNF α production was not inhibited by the addition of L-NIL or D-NIL (fig 4-11).

This suggests that TNF α does not play a major role in the induction of endothelial cell apoptosis in this co-culture assay system since apoptosis is inhibited with the addition of L-Nil despite continued TNF α production. In addition the profound reduction in MCEC-1 apoptosis (>90%) by the addition of L-NIL suggests that NO is the dominant death effector in this system.

4.7.3 Fas-L is not expressed by the activated BMDM

Using flow cytometric analysis our positive control cell KFL9 (derived from the parent cell K5625 a leukaemic lineage transfected with Fas-L) can be seen to express modest amounts of Fas-L. Analysis of BMDM indicates that FAS-L is not expressed to a significant level thereby excluding Fas-L as a major death effector in this setting (fig 4-12)

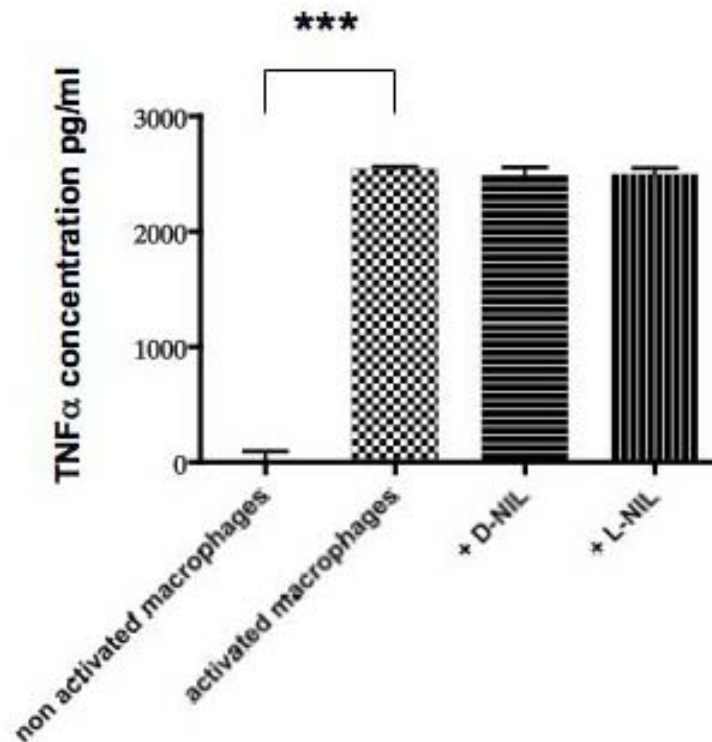


Figure 4- 11 L-Nil does not effect TNFα production by activated Mφ

Using an ELISA kit the supernatant from Mφ incubated for 24 hours with and without LPS/IFN-γ and with the addition of either L-Nil or D-Nil were tested for TNFα production. Activated Mφ produce significantly higher levels of TNFα than non activated Mφ $p < 0.0001^{***}$. This TNFα production is not inhibited with the addition of either L-Nil or D-Nil (n=3)

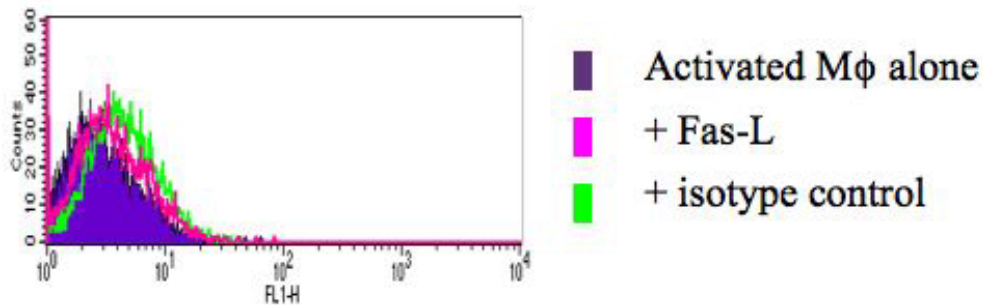
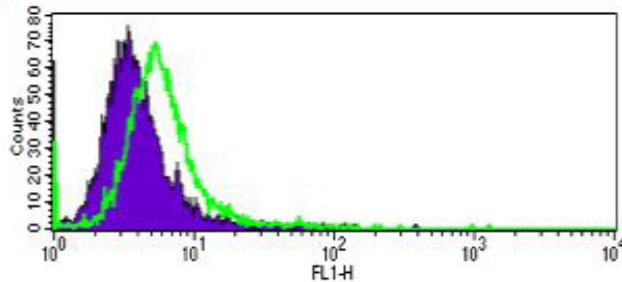
A**B**

Figure 4- 12 FAS Ligand is not expressed by bone marrow derived M ϕ

Mature BMDM were cultured for 24 hours on a 12 well plate (500,000 cells/well) activated with LPS/IFN γ . They were then scraped of the culture plate and stained with Rat anti-mouse Fas-L antibody or an isotype control Rat IgG2 followed by FITC conjugated secondary antibody prior to flow cytometric analysis. Control group of activated M ϕ with no FAS-L antibody was also analysed.

A Flow cytometry analysis demonstrates no difference in the FAS-L stained M ϕ compared to either the unstained group or the isotype control suggesting that there is no FAS-L expression by these activated M ϕ .

B The positive control KFL9 cell line demonstrates a shift to the right on the histogram compared to the control group demonstrating Fas-L expression

4.8 Summary & discussion

The *in vitro* studies aimed to dissect the mechanism of endothelial cell death by cytokine activated M ϕ . Previous work in our lab has shown using *in vitro* co-culture techniques that activated M ϕ are cytotoxic to both mesangial cells and tubular epithelial cells. In these studies the data demonstrates that activated M ϕ are directly cytotoxic to endothelial cells *in vitro* both by inducing apoptosis as well as inhibiting proliferation. However, the inhibition of proliferation was independent of cytokine activation.

M ϕ activation was achieved with the use of LPS and IFN γ . This method of activation was chosen in an attempt to mirror classical activation of the M ϕ , which would occur through IFN γ production, by the T cell. In this way I attempted to mirror an immune response *in vivo* where the T cells would activate M ϕ via IFN γ .

The endothelial cells used in this experiment were from CBA/CA x C57/BL10 strains. The M ϕ we used were from murine C57 BL/6 females.

It has been found from previous work in our group that different strain combinations did not affect the level of apoptosis of tubular cells in co-culture. In addition it is very unlikely that there is an allogeneic reaction occurring in the co-culture as no immune reactants i.e. T cells, antibodies are present. For these reasons we did not attempt alternative strain combinations in this work.

We used a cardiac microvascular endothelial cell as opposed to a renal endothelial cell because at the time of these experiments this was the only microvascular cell line available. Since completing these experiments a paper was published by Rops et al (Kid International Dec 2004), which used a glomerular endothelial cell line. I contacted the author of these studies but had no response and this cell line was not commercially available.

After first determining that our M ϕ do indeed produce NO when activated we successfully blocked this production using L-Nil, with the inactive isomer D-Nil as control. This appeared to exert a significant protective effect on the endothelial cell preventing both apoptosis and the resultant endothelial cell loss, in contrast the addition of D-Nil was not protective.

The possible involvement of additional death effectors in our system including TNF α and Fas-L was explored. Significant levels of TNF α were produced by activated M ϕ , with TNF α levels being unaffected by the addition of L-Nil. Since L-NIL produced such a profound inhibition of endothelial cell apoptosis in the presence of continued expression of TNF α it is likely that TNF α is not a key player in this co-culture system.

Fas-Ligand, another potential death effector did not appear to be expressed by our activated M ϕ .

This evidence suggested that the major death effector in our *in vitro* system was NO.

In the human setting, $\text{TNF}\alpha$ and FAS-L may have more involvement but this was not explored.

Chapter 5.

The vascular & lymphatic endothelium in experimental murine renal transplantation

5.1 Introduction

The murine model of acute renal allograft rejection was developed by my supervisor Miss Lorna Marson. The strain combination used for the allograft was donor Balb-c to FVB recipient. This provided an H2 haplotype mismatch such that termination of the experiment at day 7 produced the histological picture of acute rejection as confirmed by our renal pathologist Dr Bellamy. The Isograft control was donor FVB to recipient FVB.

Results from *in vitro* experiments described in the previous chapter indicated a key role of the M ϕ in endothelial cell apoptosis. We used a conditional M ϕ ablation model (Cailhier et al., 2005) to investigate the effect of M ϕ depletion upon the renal microvascular endothelium in the murine model of acute renal allograft rejection. Here the donor was Balb/c to recipient FVB-CD11bDTR. A single surgeon Dr F Qui with a success rate of 72% carried out all transplants.

5.2 Macrophage infiltration seen in allografts was reduced by diphtheria toxin injection

Very low numbers of F4/80 positive cells were seen in the isograft tissue. In contrast the allografts exhibited marked infiltration with interstitial F4/80 positive cells. M ϕ infiltration of the allograft was significantly reduced following DT treatment (fig 5-1). Computer image analysis was used to quantify the extent of F4/80 positive M ϕ infiltration with data being expressed as the percentage of F4/80 staining per total slide area. Interstitial M ϕ infiltration significantly increased in the medulla of the allograft compared to the isograft (0.77 ± 0.39 vs 5.77 ± 1.04 % area isograft vs allograft; $p=0.0034$). An increased infiltrate of F4/80 positive cells was also evident in the cortex although this did not reach statistical significance (fig 5.2).

With the administration of DT the F4/80 positive M ϕ infiltrate in both the cortex and medulla were significantly reduced compared to the allograft controls. Cortex (1.01 ± 0.46 vs 3.78 ± 0.78 % area, M ϕ ablation injection v control allograft; $p<0.05$). Medulla (0.77 ± 0.39 vs 2.36 ± 0.68 % area, M ϕ ablation vs control allograft; $p<0.05$) (fig 5-2) There was no significant difference in M ϕ infiltration between the isograft and the M ϕ ablated allograft.

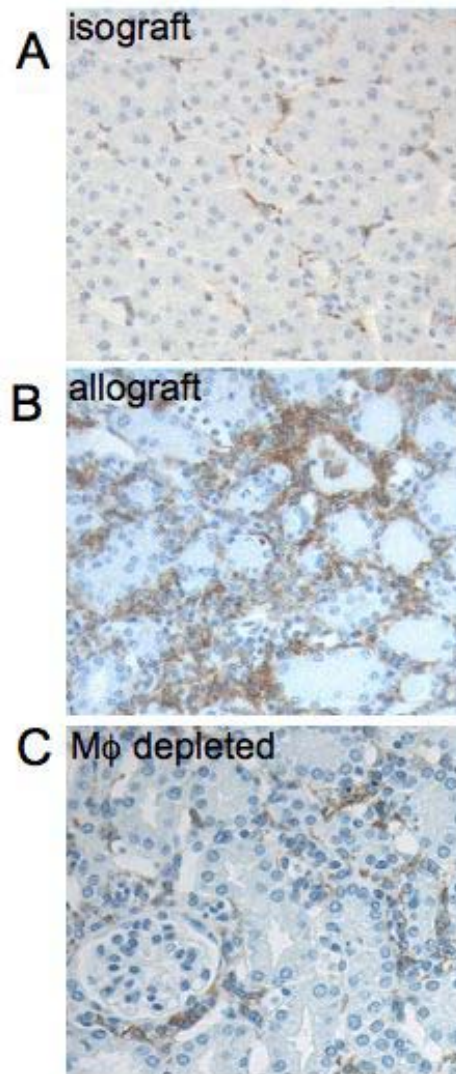


Figure 5- 1 Murine allografts exhibit increased M ϕ infiltration which is reduced following DT treatment.

F4/80 immunostaining performed in murine transplanted renal tissue harvested as day 7. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). M ϕ depleted Balbc→CD11b-DTR (n=10).

A Isografts exhibited little interstitial M ϕ infiltration. **B** Allografts exhibited large M ϕ infiltration. **C** DT treatment induced monocyte/M ϕ ablation
(x200 magnification)

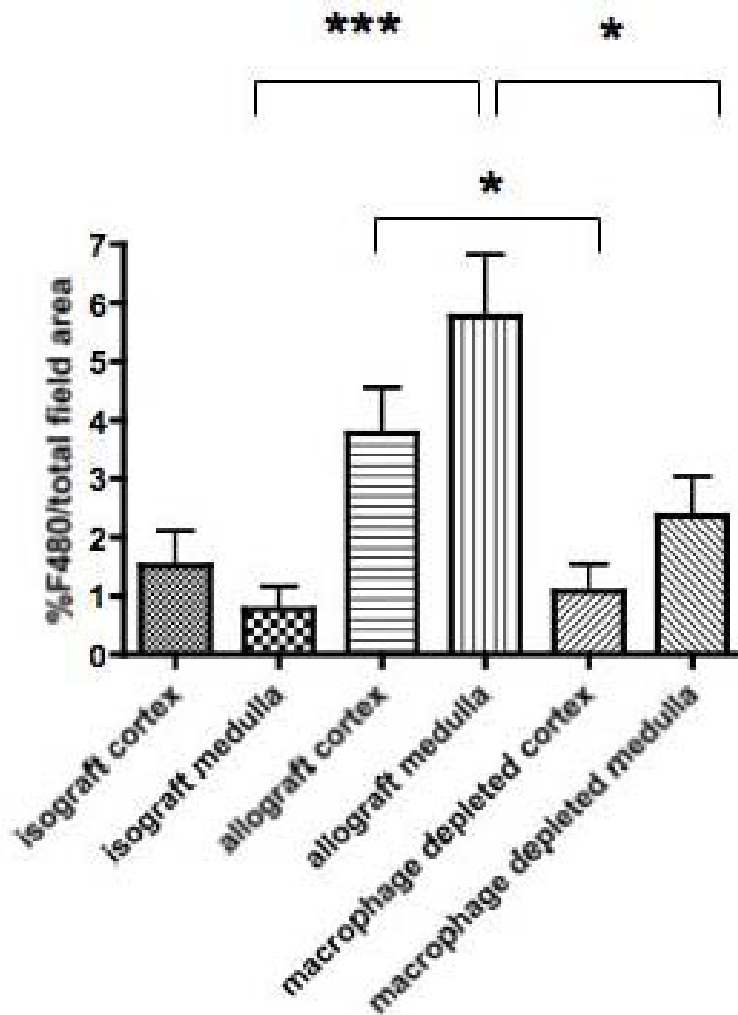


Figure 5- 2 M ϕ ablation is achieved by injection of diphtheria toxin.

F4/80 immunostaining was performed on murine transplanted renal tissue harvested at day 7 and quantified by computer image analysis. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). M ϕ depleted Balbc→CD11b-DTR (n=10).

There is significant medullary infiltration of F4/80 positive M ϕ in allografts compared to the isografts $p < 0.001^{***}$. This M ϕ infiltration is significantly inhibited both in the medulla and cortex of allografts following DT injection $p < 0.05^*$.

5.3 T and B cell infiltration was not affected by DT injection

Very little T cell infiltration was seen in the isografts. In contrast a large interstitial T cell infiltrate was evident in both the control allograft the M ϕ depleted tissue (fig 5-3). Quantifying these observations and expressing our findings as the percentage CD3 staining per field area we found almost negligible numbers of T cells in the interstitium of both the cortex (0.25 ± 0.07) and medulla (0.15 ± 0.04) of the isografts (fig 5-4)

Significant T cell infiltration was found in the allograft cortex and medulla compared to the isografts, (8.74 ± 0.76 vs 0.25 ± 0.07 % area; M ϕ depleted cortex vs control isograft cortex; $p<0.001$) (6.8 ± 0.86 vs 0.15 ± 0.04 % area M ϕ depleted medulla vs control isograft medulla; $p<0.001$).

The M ϕ depleted allografts also exhibited significantly higher T cell infiltration compared to the isograft; Cortex (7.58 ± 1.50 vs 25 ± 0.07 % area, M ϕ depleted vs control isograft; $p<0.001$), medulla (4.7 ± 1.4 vs 0.15 ± 0.04 % area; M ϕ depleted vs control isograft; $p<0.001$). There was no significant difference in T cell infiltrate between control allografts and M ϕ depleted allograft (fig 5-4)

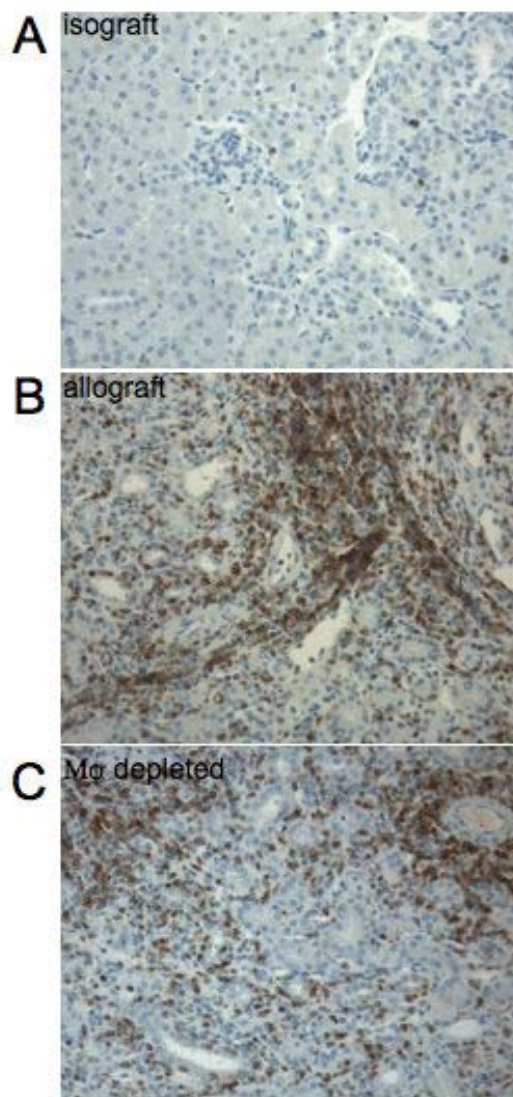


Figure 5- 3 Allografts exhibit increased interstitial T cell infiltration, which is not reduced by DT treatment.

CD3 immunostaining was performed on murine transplanted renal tissue harvested at day 7. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). Mφ depleted Balbc→CD11b-DTR (n=10).

A Isograft exhibits little interstitial T cell infiltration. **B** Allografts show a large T cell infiltrate. **C** Significant T cell infiltrate is still present following DT treatment.
(x200 magnification)

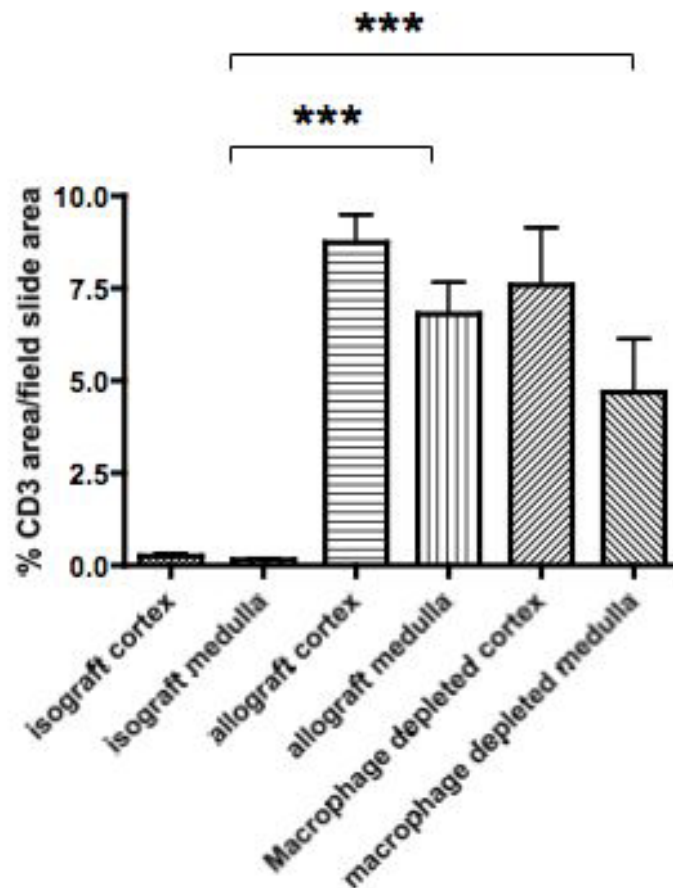


Figure 5- 4 Diphtheria toxin mediated monocyte M ϕ ablation does not affect T cell infiltration in murine renal allograft tissue.

CD3 immunostaining performed on murine transplanted renal tissue harvested at day 7. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). M ϕ depleted Balbc→DTR (n=10).

There is significant infiltration of T cells in control and M ϕ ablated allografts compared to the isografts $p < 0.001^{***}$. This T cell infiltration is not inhibited by DT injection.

Very little B cell B220 +ve infiltrate was seen in the isografts. In contrast a large interstitial B cell infiltrate comprising of both B cell aggregates and dispersed B cells was evident in the control allograft the M ϕ depleted allograft (fig 5-5). B cell infiltration was quantified by computer image analysis and the data expressed as the percentage of B220 positive immunostaining per field. Almost negligible B cell infiltrate in the isografts was found; cortex (0.009 ± 0.005 % area) medulla (0.016 ± 0.01 % area). There was a significant infiltrate seen in the allografts compared to the isograft; cortex (0.68 ± 0.15 vs 0.009 ± 0.005 % area allograft vs isograft; $p<0.001$), medulla (0.15 ± 0.03 vs 0.016 ± 0.01 % area allograft vs isograft; $p<0.05$).

B cell infiltration in control allograft and M ϕ depleted allograft were comparable (Fig 5-6)

The level of circulating mononuclear cells was determined at the end of the experiment on day 7. There was a significant reduction in circulating monocytes in the M ϕ depleted group compared to the control allografts. There was also a significant reduction in circulating B cells compared to the allografts. There was no difference in circulating CD4 or CD8 T cells between M ϕ depleted and control allografts (fig 5-7)

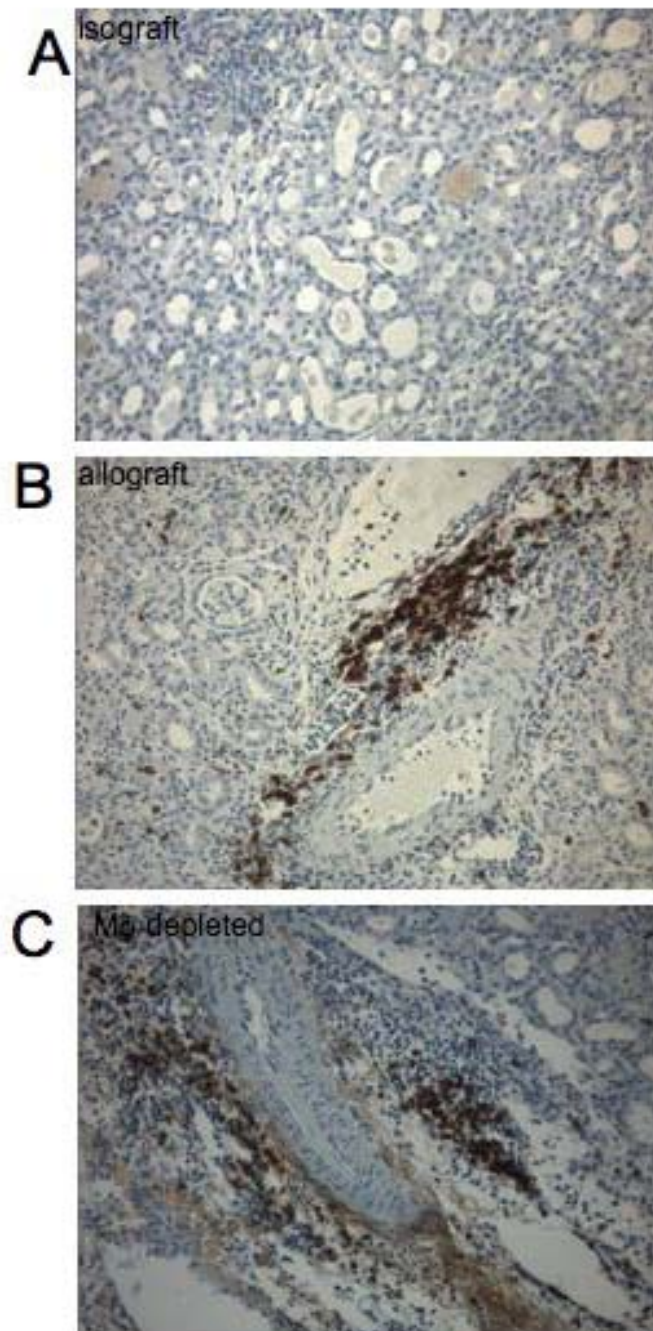


Figure 5- 5 Allografts exhibit increased interstitial B cell infiltration, which is not inhibited by DT treatment.

B220 immunostaining performed on murine transplanted renal tissue harvested at day 7. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). Mφ depleted Balbc→CD11b-DTR (n=10).

A Isograft exhibits little interstitial B cell infiltration. **B** Allograft shows a large B cell infiltrate. **C** Significant B cell infiltrate is still present following DT treatment. (x200 magnification)

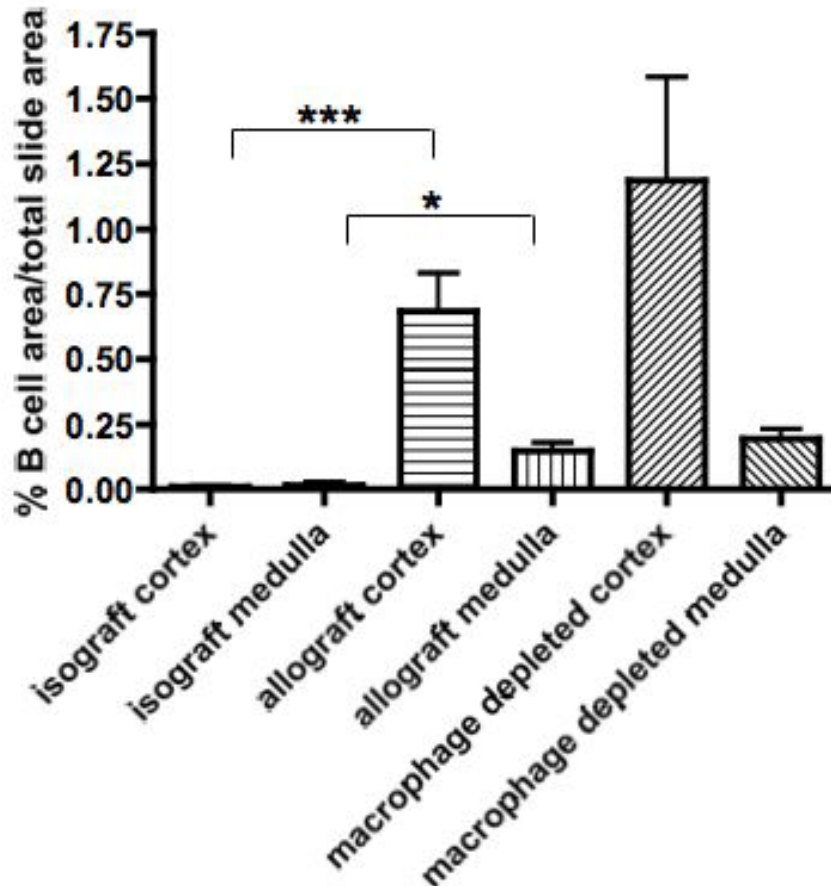


Figure 5- 6 Diphtheria toxin mediated monocyte/M ϕ ablation does not affect B cell infiltrate in murine renal allograft tissue.

B220 immunostaining performed on murine transplanted renal tissue harvested at day 7. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). M ϕ depleted Balbc→CD11b-DTR (n=10).

There is significant infiltration of B cells in the cortex $p < 0.001^{***}$ and medulla $p < 0.05^*$ of allografts compared to the isografts. This B cell infiltration is not inhibited by DT mediated monocyte/M ϕ ablation.

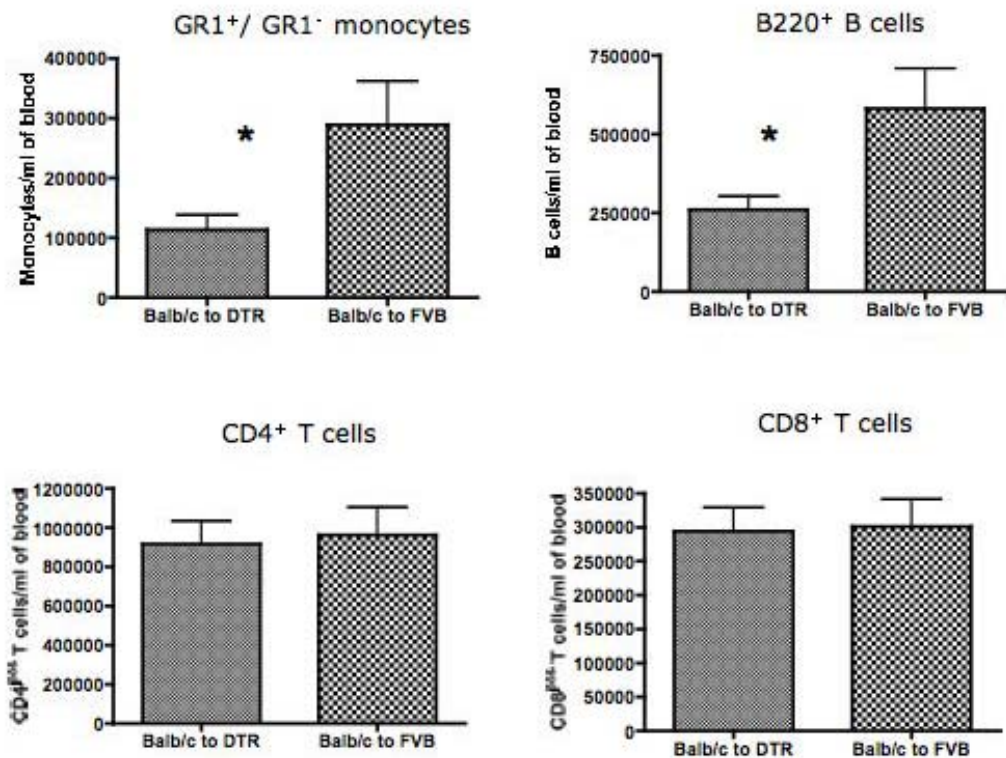


Figure 5- 7 Blood analysis of circulating mononuclear cells at day 7 post transplant using flow cytometry.

Venous blood was collected from the murine recipients at the time of termination of the experiment on day 7 post transplant. Peripheral blood leukocytes were prepared, immunolabelled and assessed by flow cytometry.

A Significant reduction in the number of circulating monocytes in the DT treated CD11b-DTR mice compared to FVB control mice $p < 0.05^*$.

B Significant reduction in the number of circulating B cells in the DT treated CD11b-DTR mice compared to FVB control mice $p < 0.05^*$.

C & D No difference in the number of circulating T cells (CD4 & CD8) in the DT treated CD11-bDTR mice compared to the FVB control mice.

(This study was carried out in our laboratory by T Kipari)

5.4 Reduction in microvasculature seen in murine allografts is inhibited by macrophage depletion

Peritubular capillaries (PTC) identified by CD31 immunostaining were present in the isografts. In the allograft there was evidence of loss of PTCs with preservation being seen following M ϕ depletion (fig 5-8). Quantifying these observations and expressing our findings as the mean number of peritubular capillaries per 100 tubules (Ohashi et al., 2002) the allografts were found to have a significantly reduced microvasculature in both the cortex and medulla compared to the isograft; cortex (64.97 ± 7.28 vs 130 ± 10 control allograft vs isograft; $p=0.002$), medulla (70.5 ± 6.9 vs 127 ± 7.3 , control allograft vs isograft; $p=0.002$). Preservation of the microvasculature was achieved with M ϕ depletion and significantly more PTCs were seen in this group compared to the allografts. Numbers of PTC seen in this M ϕ depleted group were in fact comparable to those seen in the isografts (fig 5-9)

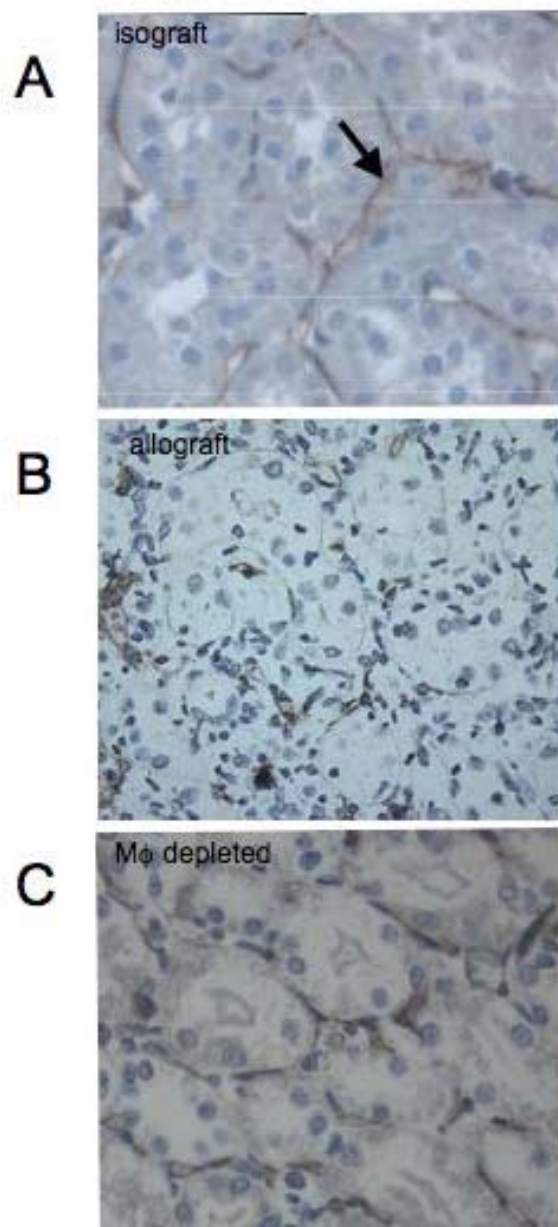


Figure 5- 8 Allograft exhibit a reduction in CD31 microvasculature, which is preserved following DT, mediated monocyte/M ϕ ablation.

CD31 immunostaining performed on murine transplanted renal tissue harvested at day 7. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). M ϕ depleted Balbc→CD11b-DTR (n=10).

A Peritubular capillaries are visible in the isograft tissue (example arrowed).

B Destruction and loss of peritubular capillaries are evident in the allograft.

C Preservation of peritubular capillaries is evident following DT treatment. (x400 magnification)

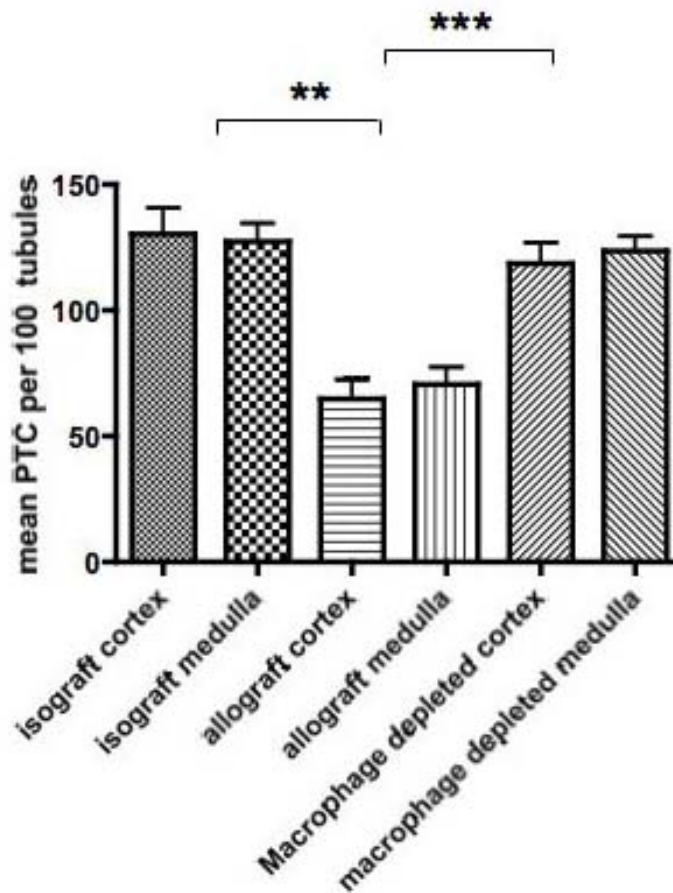


Figure 5- 9 Diphtheria toxin mediated monocyte/M ϕ ablation preserves microvasculature in murine renal allograft tissue.

CD31 immunostaining performed on murine transplanted renal tissue harvested at day 7. Isograft FVB \rightarrow FVB (n=5). Allograft Balbc \rightarrow FVB (n=18). M ϕ depleted Balbc \rightarrow CD11b-DTR (n=10).

There is significant loss of peritubular capillaries (PTC) in the cortex and medulla of allografts compared to the isografts $p < 0.01^{**}$. This PTC loss is inhibited by DT mediated monocyte/M ϕ ablation $p < 0.001^{***}$.

5.5 Reduction in tubular iNOS expression and increased interstitial iNOS expression is seen in murine allografts.

Isografts appeared to express high levels of iNOS within all tubules with very little being seen within the interstitium. In contrast allografts showed high levels of interstitial iNOS expression and loss of tubular iNOS expression. In the M ϕ depleted group there appeared to be preservation of tubular iNOS expression with a reduction in interstitial iNOS expression (fig 5-10).

Quantifying these observations and expressing the findings as the mean number of iNOS positive cells per 25-point graticle, isografts exhibited almost negligible interstitial iNOS expression, which is significantly increased in the allografts (0.07 ± 0.046 vs 5.03 ± 0.33 per 25 point graticle, isograft vs allograft; $p < 0.001$). M ϕ depleted allografts express significantly less interstitial iNOS compared to the control allograft (2.5 ± 0.33 vs 5.03 ± 0.33 per 25 point graticle, M ϕ depleted vs control allograft; $p < 0.001$) (fig 5-11A).

Expression of tubular iNOS was quantified and expressed as the mean number of iNOS positive tubules per 25-point graticle. The isografts expressed significantly higher levels of this tubular iNOS compared to allografts (8.1 ± 1.03 vs 1.84 ± 0.3 per 25 point graticle isograft vs allograft; $p < 0.001$). Although there was a non-significant trend towards preservation of tubular iNOS expression following

M ϕ depletion, there was still significantly less expression to that seen in the isografts (fig 5-11B)

5.6 Urine nitrite concentrations are comparable pre and post transplantation and are unaffected by M ϕ depletion

Urine nitrite concentrations were analysed using Griess assay and corrected for urine concentration by expressing the data as the urine nitrite concentration/creatinine ratio. Urine samples were taken by direct bladder puncture in both the allograft group and the M ϕ depletion group pre transplant and at day 7 post transplant at the time of sacrifice. This demonstrated no difference in nitrite concentrations pre and post transplant or between allograft and M ϕ depleted groups (fig 5-12).

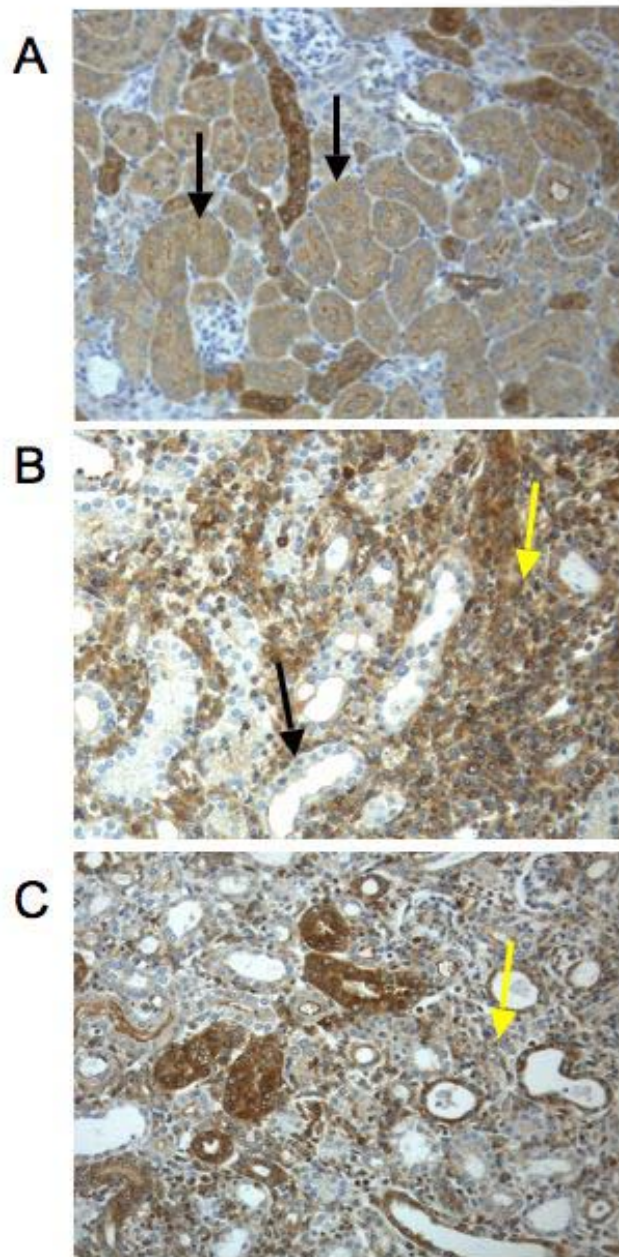


Figure 5- 10 iNOS immunostaining on murine transplanted renal tissue.

Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). Mφ depleted CD11b-Balbc→DTR (n=10).

A Isografts exhibit tubular expression (example black arrowed) of iNOS with a very low level of interstitial expression. **B** Allografts exhibit increased interstitial iNOS expression (example yellow arrow) and reduced tubular iNOS expression compared to isografts (example black arrow) **C** Mφ depletion inhibits interstitial iNOS expression in allografts (example yellow arrow). (x200 magnification)

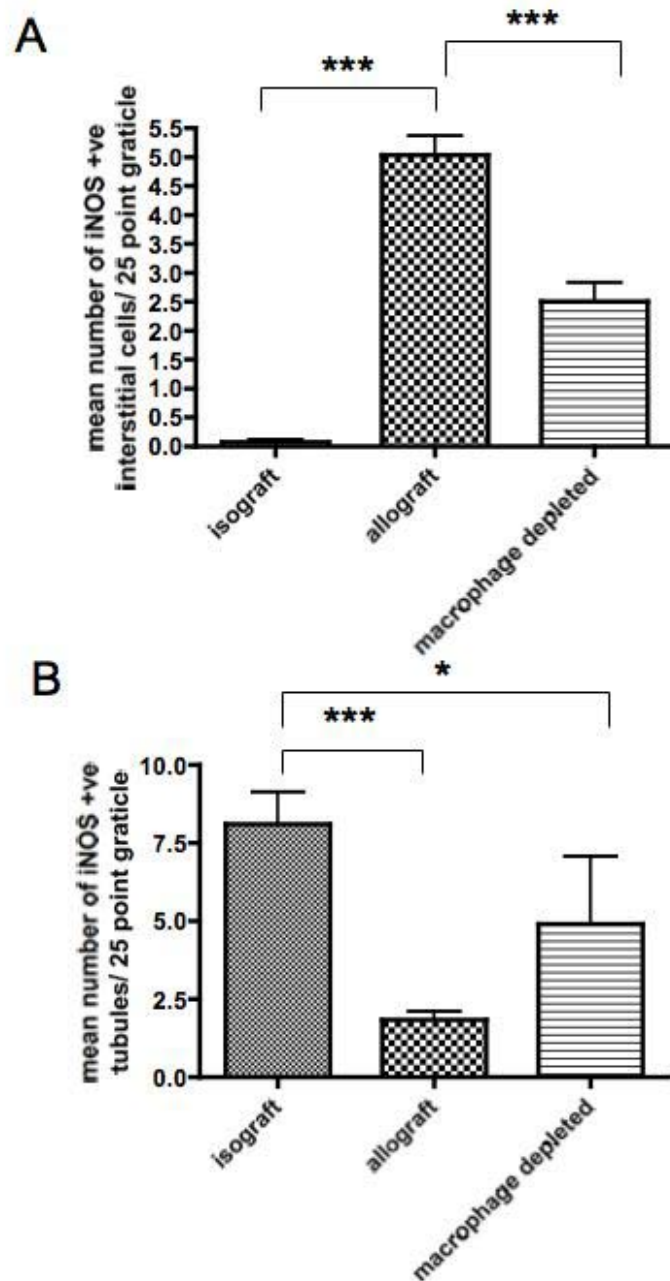


Figure 5- 11 Allografts exhibit increased interstitial iNOS expression and reduced tubular expression of iNOS compared to isografts.

iNOS immunostaining performed on murine transplanted renal tissue. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). Mφ depleted Balbc→DTR (n=10).

A There is a significant increase of interstitial iNOS expression in allografts compared to the isografts $p < 0.001^{***}$. This is inhibited by Mφ depletion $p < 0.001^{***}$

B There is significant loss of tubular iNOS expression in allografts compared to isografts ($p < 0.001^{***}$) which is not preserved with Mφ depletion ($p < 0.05^{*}$)

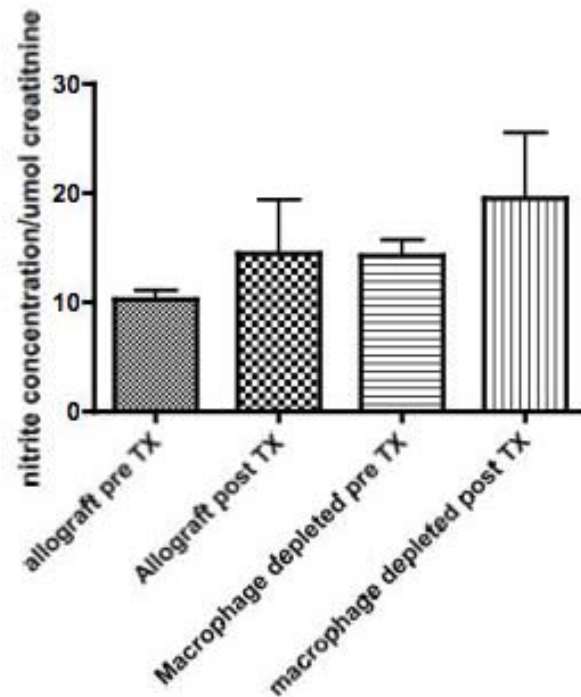


Figure 5- 12 Urine nitrite concentration is not affected by M ϕ depletion.

Urine was collected pre transplant and at day 7 post transplant in both the allograft and M ϕ depleted models. This was analysed for nitrite concentration using a Griess assay and corrected for creatinine concentration. No difference in nitrite concentration was seen following transplantation either within groups or between the control allograft and M ϕ depleted allograft.

5.7 Lymphatic vessels in murine transplanted renal tissue

In the isografts, discrete podoplanin positive lymphatic vessels were positioned around intrarenal arteries. In the allograft in addition to these discrete lymphatic profiles there was also a reticular network of podoplanin positive channels not yet clearly defined as vessels spreading out from the perivascular region. This was not seen in the isograft and was reduced in the M ϕ depleted allografts (fig 5-13).

The numbers of podoplanin positive vessels were expressed as the mean number of discrete podoplanin positive vessels, which were circular and had a visible lumen per intra renal artery. In the allograft there was a significant increase in these fully formed lymphatics compared to the isografts (4.7 ± 0.42 vs 2.5 ± 0.2 lymphatic vessels/arterial profile, allograft vs isograft; $p < 0.001$). In the M ϕ depleted allograft the lymphatic vessel number was reduced to levels comparable to those seen in the isograft (2.2 ± 0.33 vs 2.5 ± 0.2 lymphatic vessels/arterial profile, M ϕ depleted vs isograft), which is significantly reduced, compared to the allograft (fig 5-14)

An additional interesting observation was that in the allograft tissue some of these new well defined lymphatics appeared functional as they contained an abundance of cells within their lumen including mononuclear cells with ingested apoptotic cells. This was not seen in the isograft or M ϕ depleted tissue (fig 5-15)

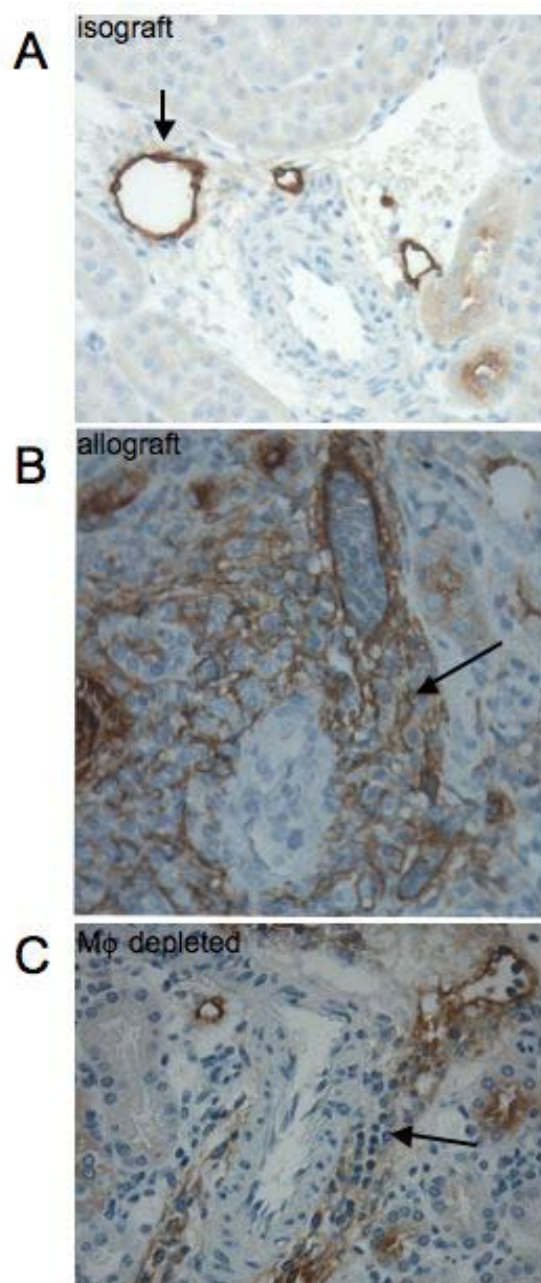


Figure 5- 13 Podoplanin positive vessels in murine transplanted renal tissue.

Podoplanin immunostaining was performed on murine transplanted renal tissue harvested at day 7. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). Mφ depleted Balbc→CD11b-DTR (n=10).

A Isografts exhibit a normal number of well defined perivascular lymphatic vessels (example arrowed).

B Allografts develop a reticular network of developing podoplanin positive lymphatics (example arrowed).

C DT mediated monocyte/Mφ ablation treatment inhibits the development of this podoplanin positive network (example arrowed)(x200 magnification)

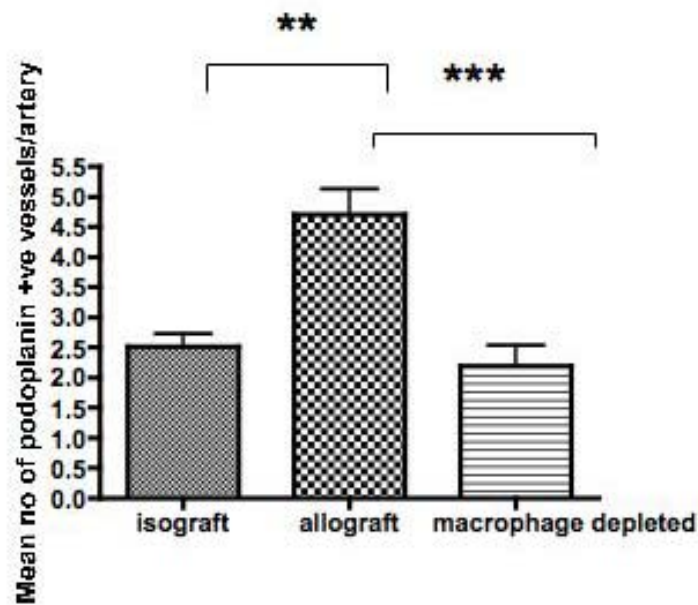


Figure 5- 14 The increased number of lymphatic venules evident in allografts is inhibited following M ϕ depletion.

Podoplanin immunostaining was performed on murine transplanted renal tissue harvested at day 7. Isograft FVB→FVB (n=5). Allograft Balbc→FVB (n=18). M ϕ depleted Balbc→CD11b-DTR (n=10).

There is a significant increase in lymphatic vessels in allografts ($p < 0.01^{**}$) compared to the isografts ($p < 0.05^{*}$). This is significantly inhibited in the M ϕ depleted allograft ($p < 0.001^{***}$)

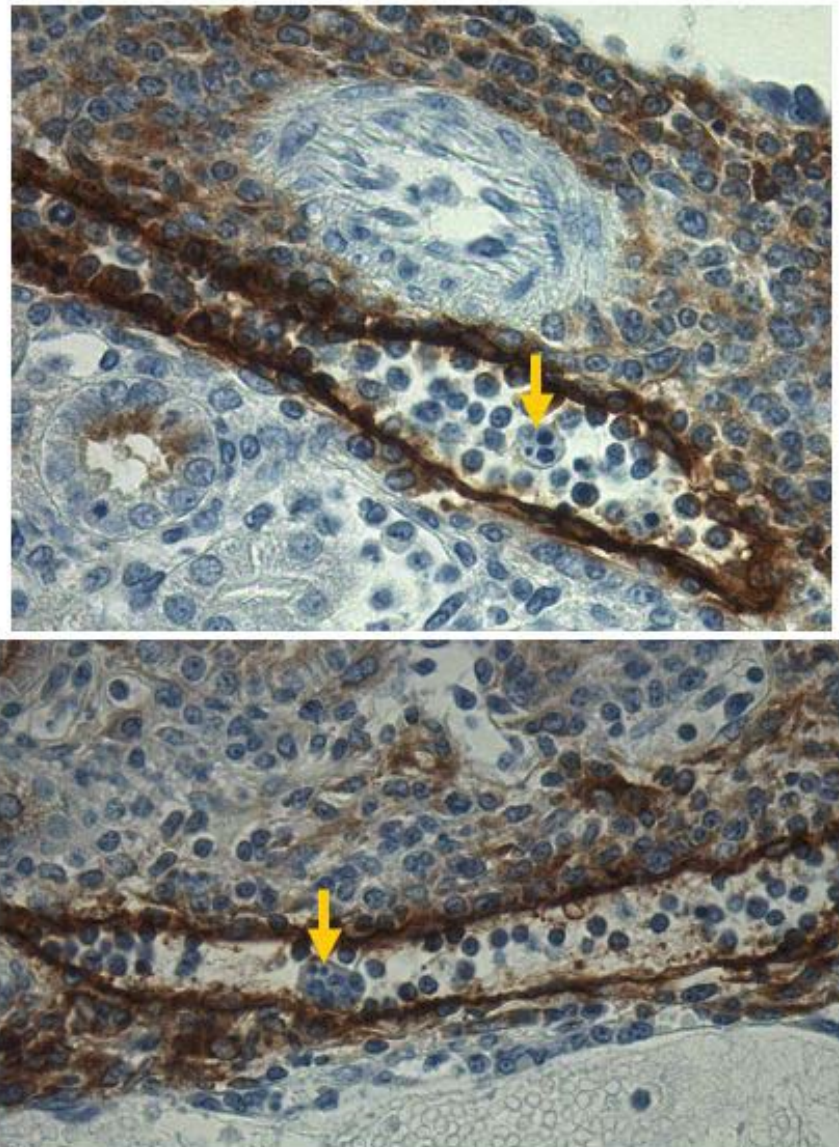


Figure 5- 15 Mφ containing an ingested apoptotic cell within a podoplanin positive lymphatic vessel arrowed).

Murine renal allograft Balb→FVB harvested at day 7 and immunostained for podoplanin lymphatic marker.
(x400 magnification)

Within the podoplanin +ve vessels are both Mφ containing apoptotic cells, as well as numerous other cells, which implies functionality of these podoplanin +ve vessels.

5.8 Summary & discussion

To summarise, a reduction in interstitial microvasculature in the allograft compared to the isograft controls was found associated with increased interstitial iNOS expression. In addition, there was an increase in lymphatic vessel number in the perivascular region. All these changes were inhibited following M ϕ depletion.

In this thesis all the data was carried out on the model of acute rejection in which the recipient retained a native functioning kidney and therefore graft function cannot be determined.

In this model a loss of tubular iNOS expression was demonstrated which was previously suggested as being protective (Du et al., 2006) and an increase in interstitial iNOS expression, thought to be detrimental was evident in the allograft compared to the isograft.

M ϕ depletion significantly reduced interstitial iNOS expression compared to the allograft and although there was a non-significant trend towards preservation of tubular iNOS expression following M ϕ depletion, there was still significantly less expression to that seen in the isografts.

Interestingly there was significant inhibition of tubular iNOS expression in the allograft compared to the isograft. Recent work suggests that endogenous iNOS expression by tubular cells at low concentrations has a protective role in renal grafts and therefore these findings that tubular expression is reduced in the allograft where microvascular loss is seen is consistent with this.

This finding of significant tubular iNOS expression in the isograft, which then appears to be inhibited and replaced, by interstitial expression may explain the urine nitrite findings in this model. Previous studies have demonstrated increased urinary nitrite in rejecting grafts compared to those who continue to enjoy stable function (Smith et al., 1996). In these studies no difference was found between pre and postoperative concentrations when corrected for creatinine concentration within groups or between the two groups. I speculate that this is due to a significant loss of tubular iNOS expression at the same time as there is an increased interstitial expression and therefore the overall nitrite produced remains constant i.e. comparable amounts of NO continue to be produced but the source varies i.e. from tubular to interstitial with tubular NO generation being predominant in isografts and interstitial NO generation being predominant in allografts.

In the isograft there is generally less interstitial infiltrate, which mirrors the low interstitial iNOS expression.

Using immunostaining renal tubules are seen to produce iNOS, which is reduced in the allograft, this may be due to the tubular epithelial destruction, or there is down regulation of tubular iNOS expression in the allograft compared to the isografts.

Despite evidence of a new network of lymphatics spreading out towards the interstitium no well defined lymphatic vessels were seen in the interstitium: unlike the human CAN tissue. This may be

because the murine model is looking at acute rejection whereas the human tissue is CAN and so we may be seeing the process at a much earlier time point. Alternatively the pattern may vary between species.

The reticular network of podoplanin stain seen in the murine allograft may be immature lymphatics forming. Alternatively it may be that infiltrating M ϕ may express podoplanin since M ϕ have been documented to transdifferentiate to LEC. Double staining with F4/80 and podoplanin would be informative as would an additional LEC marker.

Could all the changes observed in the murine model be purely due to non-specific inflammation or are the changes specific to graft rejection? It does not appear to be just a response to renal IRI because the isograft has presumably endured a comparable degree of IRI as the allograft. Thus the mononuclear cell infiltrate, loss of microvasculature and increased lymphatic number are specific to graft rejection.

We found a significant interstitial infiltrate of both B and T cells in the allograft compared to the isograft and importantly this infiltration was not attenuated by DT treatment. Examining the blood of these mice at day 7 there was a reduction in CD11b/GR1⁺ and GR1⁻ monocytes. No change was found in circulating T cells supporting our findings in the renal tissue, however there was a significant reduction in circulating B cells not seen in the tissues. We know B

cells may express CD11b. We also know that other forms of M ϕ ablation such as clodronate can also have an effect on lymphoid cells. We could use these differences to our advantage in the future to tease out mechanisms. In addition we could carry out pure B cell depletion using the murine antibody similar to Rituximab to study the role of B cells in rejection.

Further work looking at the effect of DT treatment on the dendritic cell (DC) population was carried out in the laboratory after I had completed my research period. It was found that CD11C⁺ DC infiltration was reduced in the renal allograft which would be expected as GR1⁺ monocytes differentiate into DCs in the tissue, therefore if you knock out all monocytes within the circulation this will result in a reduction of cells available to develop into DCs. Importantly it was found that there was no reduction in splenic CD11c⁺ cells following DT treatment.

Consultant Renal pathologist Dr Chris Bellamy assessed the severity of acute rejection in the renal sections. He found only mild inflammation in the isografts, evidence of both cellular and vascular rejection in the allografts with a trend towards reduction in both processes in the DT treated group compared to the allografts.

Chapter 6.

Lymphatic phenotyping and isolation *in vitro*

6.1 Background

The studies outlined in previous chapters on both human and murine tissue using immunostaining demonstrated the existence of two endothelial cell populations: vascular and lymphatic. Studies in human CAN and a murine model of acute rejection, demonstrate that these two populations appear to respond differently to the process of CAN and acute rejection since in the presence of a M ϕ infiltrate the microvessels undergo destruction whilst lymphatic vessels increase in number. The *in vitro* work described further investigated the effect of the cytokine activated M ϕ on a population of endothelial cells and observed an increase in apoptosis of these cells.

The logical next step was to study the effect of the M ϕ on the lymphatic endothelial cell *in vitro* to examine any differences in response that may occur to that seen with the MCEC-1 cells.

For these studies to be undertaken a pure population of LECs was first required. The following chapter describes attempts to isolate and phenotype a LEC population from a mixed population of human endothelial cells.

6.2 Human umbilical vein endothelial cells (HUVECs) contain a small proportion of lymphatic endothelial cells.

Using flow cytometry studies confirmed that HUVECs were positive for the endothelial cell marker CD31. Staining with the lymphatic endothelial cell marker VEGFR-3 revealed that HUVECS also contained a small subpopulation of cells that were VEGFR-3⁺ (11.75%) (fig 6-1)

Further phenotyping using immunofluorescence confirmed that these HUVECs did indeed have a population of VEGFR-3⁺ cells (fig 6-2)

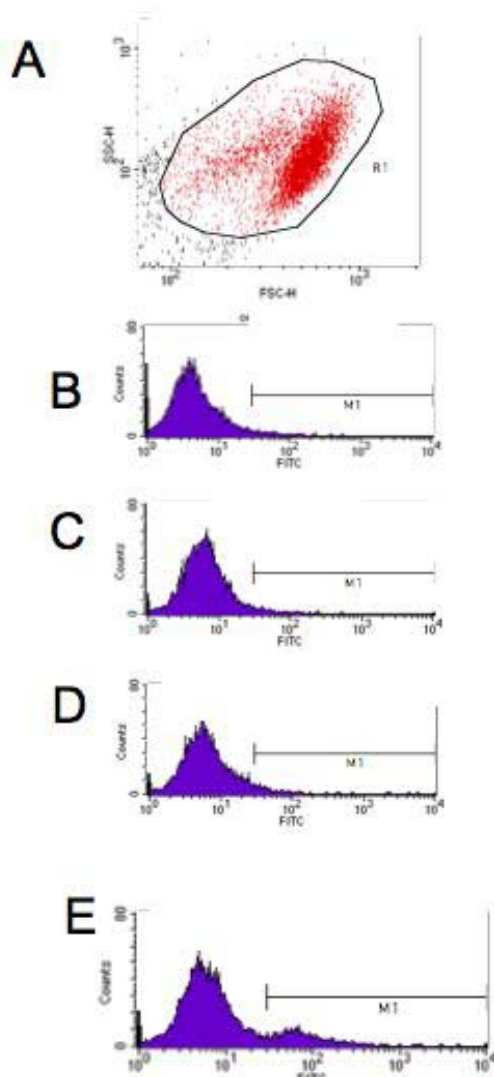


Figure 6- 1 Phenotyping HUVECs using flow cytometry

HUVECs were stained with the lymphatic endothelial cell marker VEGFR-3 with the secondary conjugated to Alexa 488. The level of expression was analysed using flow cytometry.

A the dot plot shows the total cell population

Histograms of control groups **B** Cells alone **C** VEGFR-3 secondary antibody alone +488 **D** VEGFR-3 isotype control +488.

E VEGFR-3-488 staining; histogram shows a discrete population made up of 11.75% of the total population that are VEGFR-3⁺

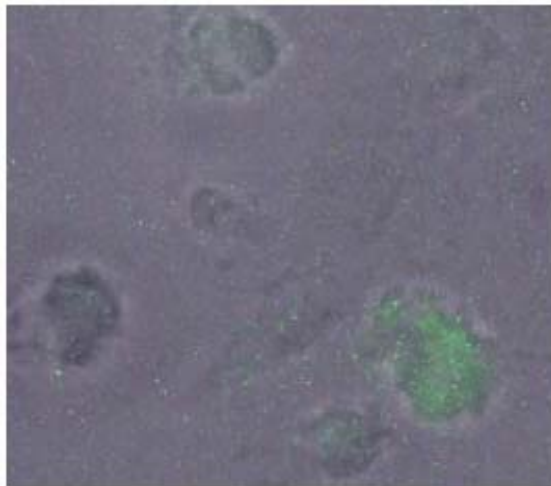


Figure 6- 2 Phenotyping HUVECs using immunofluorescence

HUVECs were prepared and labelled with VEGFR-3 and secondary antibody conjugated with Alexa 488. Analysis using fluorescent microscopy revealed some VEGFR-3-FITC (green) HUVECs.

Controls including cells alone, secondary alone (goat anti rabbit-488) and Rabbit IgG isotype control confirmed that non specific staining was not occurring. (x320 magnification)

6.3 Human dermal microvascular endothelial primary cells (HUDMECS) are made up of lymphatic and vascular endothelial cell populations.

Using flow cytometry, HUDMECs were confirmed to be positive for the endothelial cell marker CD31. Double staining with CD31 and the lymphatic endothelial cell marker podoplanin revealed that these cells were made up of two population's CD31⁺/Podoplanin⁻ cells (43.3%) and CD31⁺/podoplanin⁺ cells (56.7%) (fig 6-3).

Further phenotyping using immunofluorescence revealed that these HUVECs did indeed have a population of VEGFR-3⁺ cells (fig 6-4)

No further investigations were carried out on these primary cells as despite the claims of the manufactures they were extremely difficult to passage making it impossible to expand the cell culture in order to carry out further experiments.

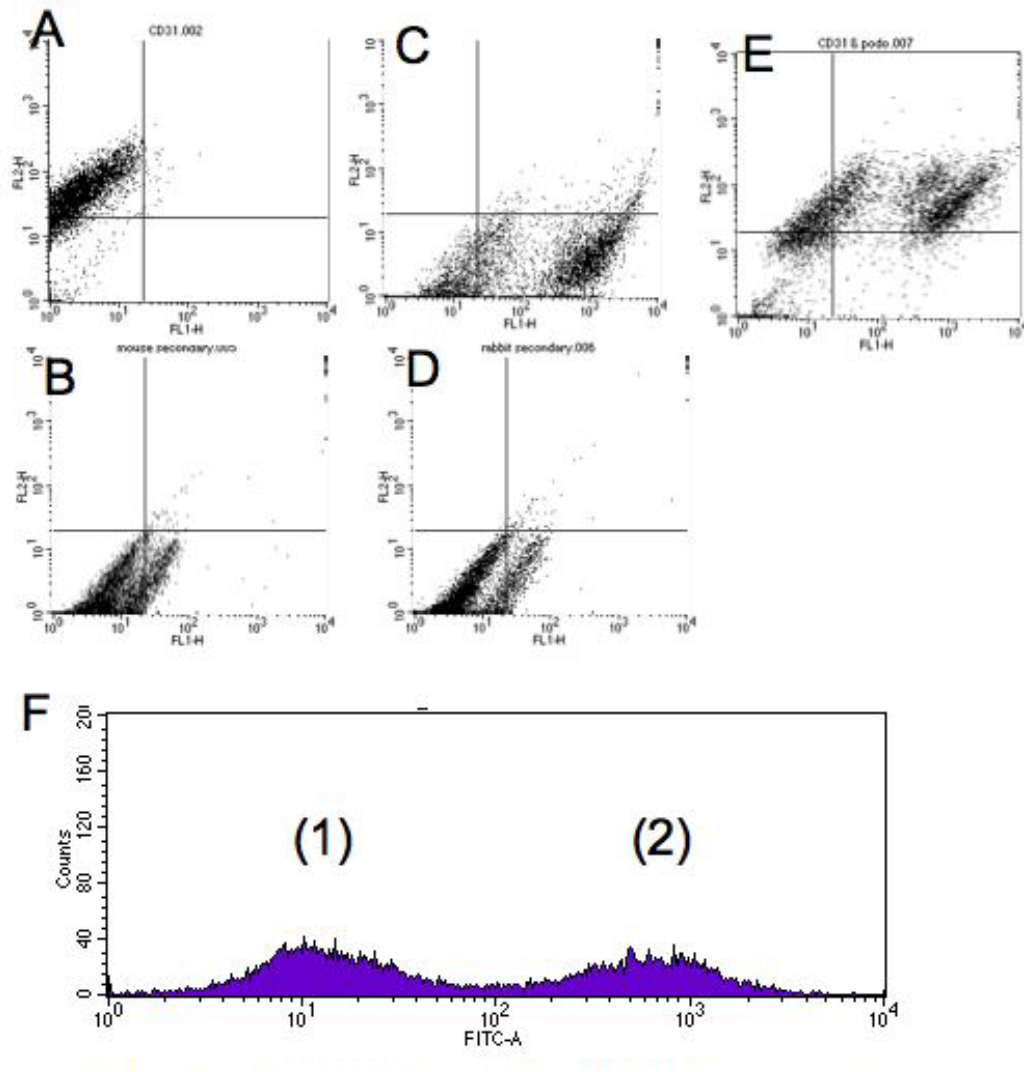


Figure 6- 3 Phenotyping HUDMECs (cell line) using flow cytometry

HUDMECs were stained with the endothelial cell marker CD31 conjugated to PE and lymphatic endothelial cell marker podoplanin conjugated to Alexa 488. The level of expression was analysed using flow cytometry. Dot plots **A** CD31 stain all cells seen in the left upper quadrant. **B** CD31 isotype control no shift seen. **C** Podoplanin stain two populations seen, one population does not shift the other shifts to the right lower quadrant. **D** Podoplanin isotype control no shift seen. **E** Double stain CD31 and podoplanin, two populations seen one in the right upper quadrant and one in the left upper quadrant.

F Histogram confirms two populations;
 (1) CD31⁺/podoplanin⁻, (2) CD31⁺/podoplanin⁺

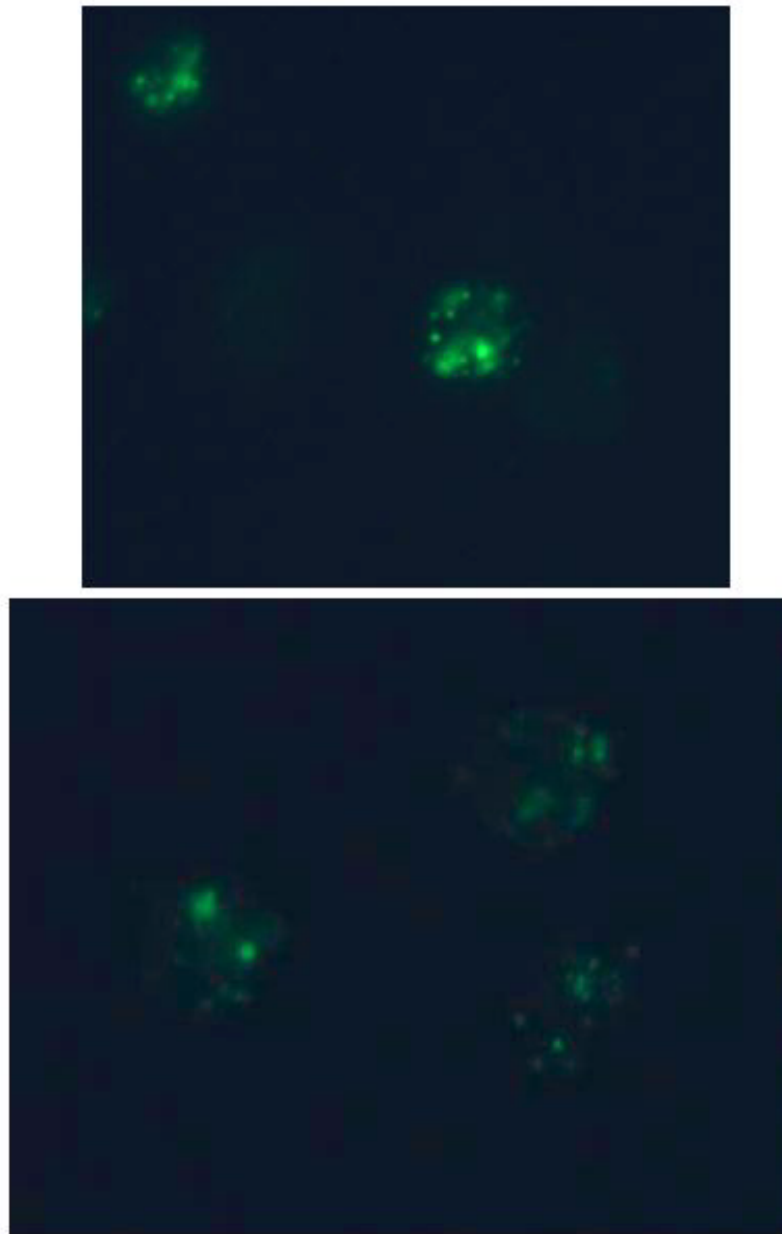
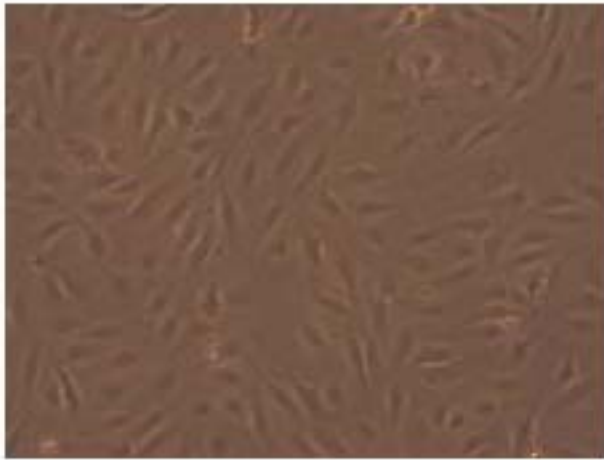


Figure 6- 4 Phenotyping HUDMECs (primary cells) using immunofluorescence.

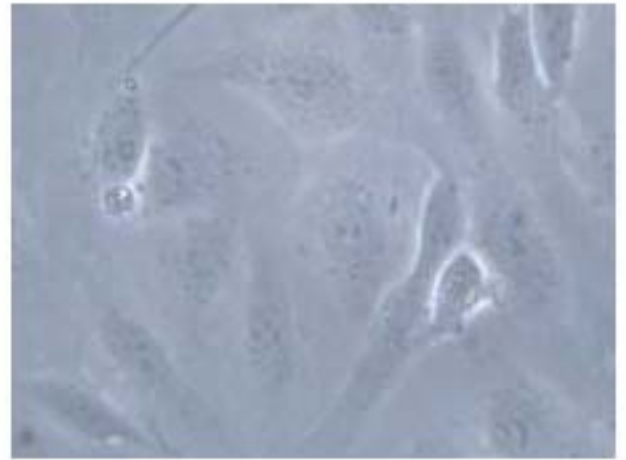
HUDMECs were prepared and labelled with VEGFR-3 and secondary antibody conjugated with Alexa 488 (green). Analysis using fluorescent microscopy revealed VEGF-3-488 positive cells (green) Controls; cells alone, isotype control and secondary antibody alone showed no staining. (x320 magnification)

6.4 Human dermal microvascular endothelial cell Line (HUDMECS)

A population of podoplanin positive HUDMECs was isolated using magnetic bead sorting. This population was successfully cultured (fig 6-5), and then phenotyped. Flow cytometry revealed that this population of HUDMECs which were 92.9% CD31⁺/podoplanin⁺ (fig 6-6). Electron microscopy studies revealed that this population of cells were non descript in ultrastructural terms with no Weibel Palade bodies and no other features such as caveolae, microvilli or multivesicular bodies (fig 6-7).



x200



x400

Figure 6- 5 HUDMECs were cultured following magnetic bead sorting for CD31⁺/podoplanin⁺ cells

Following magnetic bead sorting the cells were plated onto 48 well plates in endothelial cell medium and cultured at 37°C. After 24 hours they were analysed using light microscopy. The cells were seen to have plated down successfully and a cobble stone appearance could be seen.

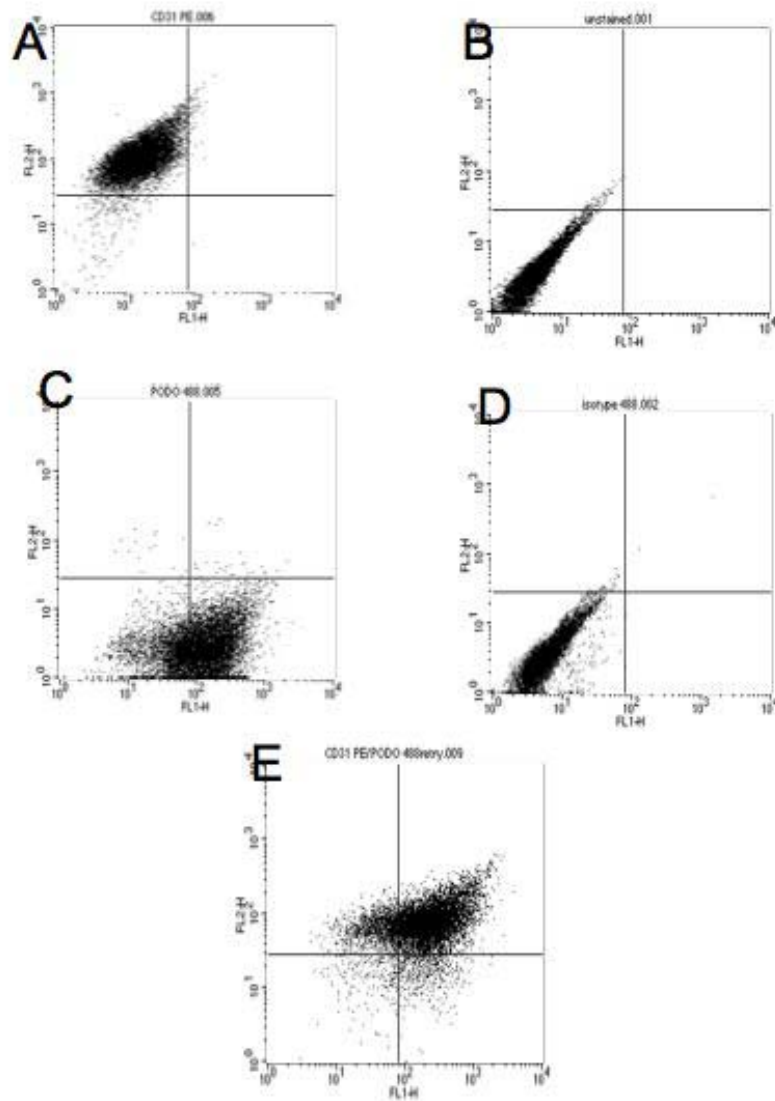


Figure 6- 6 Phenotyping HUDMECs (cell line) post magnetic bead sort using flow cytometry.

Following positive selection for CD31⁺/podoplanin⁺ cells using magnetic bead sorting the HDMECs were stained with the endothelial cell marker CD31 conjugated to PE and lymphatic endothelial cell marker podoplanin conjugated to Alexa 488. The level of expression was analysed using flow cytometry dot plots; **A** CD31 stain all cells seen in left upper quadrant. **B** CD31 isotype control no shift seen. **C** Podoplanin stain population seen in right lower quadrant. **D** Podoplanin isotype control; no shift seen. **E** Double stain CD31 and podoplanin majority of cells seen in right upper quadrant, 92%

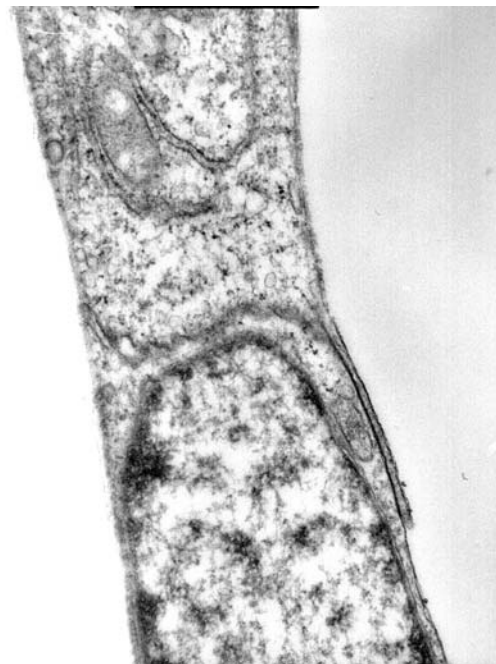
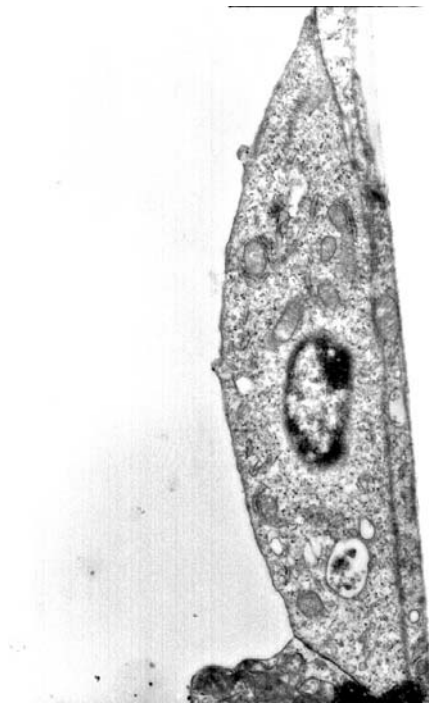


Figure 6- 7 Electron microscopy of HUDMECs (cell line) after magnetic bead sorting for podoplanin⁺ cells

6.5 Summary & discussion

The aim of this work was to obtain a pure population of human lymphatic endothelial cells, which could then be used in co-culture experiments with human M ϕ . Our prediction being that we would observe increased proliferation, increased cell number and no apoptosis.

These studies were limited by various problems. Initial studies looked at HUVECs. We know from the literature that passaging vascular endothelial cells can induce transdifferentiation into lymphatic endothelial cells (Groger et al., 2004). Our studies supported this by finding VEGFR-3 positive cells, but as expected this made up only a small proportion of the cell population 11.75%. This was therefore not a feasible source for future studies.

Further experiments used primary HUDMECs produced commercially from Promocell. Despite demonstrating that 50% of these cells were made up of Podoplanin positive endothelial cells these cells were difficult to use. There were problems with infection and the cells were only viable up to passage 3, which made them potentially expensive.

Later experiments used a cell line given to us as a gift from the Centre for Disease Control and Prevention, Atlanta, Georgia. These HUDMECs were more amenable to studies. There were no infection problems and they could be passaged up to P9. Studies demonstrated a large population of podoplanin positive endothelial

cells and these were sorted using magnetic beads. These sorted cells appeared to survive well in culture. Electron microscopy of these sorted cells revealed very non-specific structures with no ultrastructural features of vascular endothelial cells.

Further phenotyping using alternative LEC markers is necessary before experiments involving co-culture are to be developed.

Chapter 7.

Discussion

7.1 General Discussion

Introduction

Renal transplantation is the treatment of choice for patients with end stage renal failure. One of the main obstacles facing the transplant community is graft rejection. The development of effective treatment strategies to combat T cell mediated injury have significantly reduced the incidence of acute rejection with graft survival rates now reaching over 90% at 1 year following transplantation. Unfortunately rejection episodes can still complicate the early post-transplant period resulting in both early graft failure or acting as a significant risk factor for the development of late graft failure. Indeed chronic rejection and long term graft survival rates remain problematic with no effective treatment options currently available. There is still controversy and flux over the nomenclature in this area. CAN replaced the term chronic rejection in 1991 (Solez et al., 1993) and helped to address the misconception that late scarring of the graft was only due to alloimmune injury. There have now been thousands of Pubmed citations using the term CAN. However the most recent Banff conference (July 2005) decided to discontinue use of this term due to the misconception that CAN is a specific disease entity rather than a term for non-specific fibrosis. The term CAN has now been replaced by the following description "Interstitial fibrosis and tubular atrophy, no evidence of any specific aetiology" (Solez k, Am J T 2007). However for the purposes of this thesis I will continue to use

the term CAN as this was the term in use when these studies commenced.

Much of the focus in the last 15-20 years has been on the adaptive immune response with T cells being the main focus of interest. There is however evidence to suggest that the innate immune response plays a significant role in the process of allograft rejection (Land, 2007; LaRosa et al., 2007) and the work outlined in this thesis has focussed on the M ϕ and endothelial cells. The M ϕ is a versatile cell and performs diverse functions ranging from phagocytosis to antigen presentation. M ϕ are involved in the inflammatory response to injury or infection, tissue repair and importantly are key to both the innate and acquired immune response. Previously published studies indicate that M ϕ are involved in both acute and chronic rejection of the kidney and other organs (Crocker et al., 1996; Jose et al., 2003; Ozdemir et al., 2002; Pilmore et al., 2000; Szabolcs et al., 1996). In addition, more recent studies have suggested a role for M ϕ in the lymphangiogenesis that may be evident in renal allograft rejection (Kerjaschki et al., 2004), tumour development (Schoppmann et al., 2002) and other inflammatory states (Pepper et al., 2003; Skobe et al., 2001).

The focus of this work has been the innate immune response with an emphasis upon the effect of the M ϕ on both the vascular and

lymphatic endothelial cell populations in the context of both CAN (human studies) and acute rejection (murine studies). In addition, *in vitro* studies were employed to gain mechanistic insight into the cytotoxic action of M ϕ upon vascular endothelial cells. This thesis set out to investigate the hypothesis that M ϕ are directly responsible for VEC death through the production of various death effectors such as NO but play a key role in rejection associated lymphangiogenesis through the expression of lymphangiogenic growth factors such as VEGF-C. In this chapter I shall outline the key findings of my studies and suggest additional work that may be undertaken to further define the role of M ϕ in the acute and chronic complications of renal transplantation.

SUMMARY OF KEY FINDINGS

M ϕ and CAN in human transplant nephrectomies

Human tissue from transplant nephrectomies performed because of CAN were studied and compared to control kidney tissue obtained from nephrectomy specimens performed for renal tumours. Previous work from the laboratory demonstrated a reduction in the number of CD31⁺ PTC in human CAN tissue compared to controls (Adair et al., 2007). This reduction in PTC number was associated with an increased interstitial M ϕ infiltrate in the CAN tissue with a sub-population of interstitial M ϕ expressing iNOS. No obvious reduction in tubular expression of VEGF-A was evident whilst B cells exhibited

significant VEGF-A expression. Furthermore, CAN tissue exhibited interstitial lymphatic vessels that were not evident in control tissue with occasional CD68⁺ M ϕ expressing VEGF-C.

M ϕ , PTC rarefaction and lymphangiogenesis in experimental murine allograft rejection

In the murine model of acute rejection IHC studies demonstrated an increase in interstitial M ϕ infiltration in the allograft associated with a significant reduction in the number of PTC. Also, increased interstitial iNOS expression was evident compared to the isograft. M ϕ ablation undertaken from day 3 in this model was protective and inhibited the loss of PTC with PTC number in the M ϕ ablated group being comparable to that of isograft controls. In addition, M ϕ ablation was associated with reduced interstitial iNOS expression. This murine model of acute renal rejection also demonstrated a significant increase in the number of podoplanin⁺ perivascular lymphatic vessels compared to isograft controls with this increase being prevented by M ϕ ablation. In addition, a reticular podoplanin⁺ network was noted.

In vitro studies of M ϕ and endothelial cells

Co-cultures of murine M ϕ (BMDM) and vascular endothelial cells were used to examine the cytotoxic mechanisms employed by cytokine activated M ϕ in the induction of endothelial cell death. Significant levels of apoptosis of murine endothelial cells was evident

in co-cultures with cytokine activated BMDM (LPS and IFN- γ). In contrast, M ϕ -mediated endothelial cell proliferation was inhibited by both cytokine-activated and control non-activated M ϕ . M ϕ -induced apoptosis was effectively inhibited by blocking the action of the enzyme iNOS thereby supporting a key role for NO in this system. Furthermore, iNOS blockade of cytokine-activated M ϕ did not affect the marked production of TNF α by these inflammatory cells. Lastly, in somewhat preliminary work, the use of LEC markers (podoplanin and VEGFR-3) indicated that human cell populations viewed as 'vascular endothelial cells' (primary HUVECs and the HDMEC cell line) actually consist of a mixture of vascular and lymphatic endothelial cells as a distinct population of podoplanin⁺/CD31⁺ cells was demonstrated by flow cytometry. Also, immunomagnetic bead sorting of mixed cell populations produced a >90% podoplanin⁺/CD31⁺ cell population that was successfully plated and cultured with EM studies demonstrating cells with no ultrastructural features of vascular endothelial cells.

Discussion

There is still controversy in the literature as to whether increased angiogenesis or loss of microvasculature occurs during renal disease and renal allograft rejection. Ozdemir et al. demonstrated increased angiogenesis associated with increased VEGF-A expression by infiltrating M ϕ , which increased the risk of graft loss (Ozdemir et al.,

2002). However Ishii et al. demonstrated loss of PTC which correlated with both the severity of CAN and loss of graft function (Ishii et al., 2005). Work by Kang et al. and Ohashi et al. in rat and murine models of renal disease respectively also demonstrated PTC loss in native kidneys following the induction of renal inflammation and scarring with PTC loss being associated with reduced endogenous expression of VEGF-A and M ϕ infiltration (Kang et al., 2001a; Kang et al., 2001b; Ohashi et al., 2002).

Our work demonstrated a loss of the peritubular microvasculature in human tissue affected by CAN as well as murine renal allografts exhibiting rejection with both human and murine tissue exhibiting significant M ϕ infiltration. In human CAN tissue, interstitial VEGF-A expression was demonstrated but double labelling studies indicated that VEGF-A was not expressed by infiltrating M ϕ as seen by Ozdemir et al. but was prominently expressed by B cells. Since the angiogenesis described by Ozdemir et al. was based upon use of the endothelial marker CD31 that may be expressed by vascular and lymphatic endothelial cells, it is possible that the 'angiogenesis' evident reflected the net effect of an increase in LEC number and a reduced VEC number (Matsui et al., 2003; Ozdemir et al., 2002). However, this is not the case in the work described in this thesis which also used the endothelial cell marker CD31 as a reduced number of CD31⁺ vessels was demonstrated despite the presence of

increased numbers of interstitial lymphatic vessels demonstrated using the LEC marker podoplanin.

It may well be the case that in the early stages of tissue injury there is an increase in VEC number associated with increased vascular endothelial cell proliferation secondary to increased VEGF-A production by the local tissues as a response to hypoxia (Pilmore et al., 1999). However, as the injurious process continues and tissue destruction overrides the reparative response there will be a reduction in the tissue available to produce the angiogenic factors. In addition, microvascular loss may be promoted by the increased influx of M ϕ that can indirectly inhibit VEGF-A production from resident renal cells (Kang et al., 2001a; Kang et al., 2001b) and are directly cytotoxic to the endothelium.

Our finding that B cells exhibit marked expression of VEGF-A in human kidney tissue affected by CAN is of interest as recent work suggests that B cells expressing VEGF-A are important in lymphangiogenesis (Angeli et al., 2006) and VEGF-A is implicated in both angiogenesis and lymphangiogenesis. For example, Cursiefen et al. demonstrated that M ϕ are key players in the haemangiogenesis associated with ocular inflammation as they are the only source of VEGF-A (Cursiefen et al., 2004). In contrast, parenchymal cells of the kidney i.e. podocytes and tubular cells express VEGF-A and

angiogenesis may be reduced during renal inflammation as a result of M ϕ -mediated inhibition of VEGF-A expression (Kang et al., 2002; Masuda et al., 2001; Ohashi et al., 2000; Ohashi et al., 2002).

It is important to note, however, that kidneys affected by CAN exhibited reduced PTC numbers despite the presence of B cells expressing VEGF-A.

The situation is undoubtedly complex as M ϕ undoubtedly exist as a heterogeneous population within tissues with both pro-inflammatory and anti-inflammatory M ϕ being present at the same time. In addition, M ϕ may be pro-angiogenic and anti-angiogenic (Ozdemir et al., 2005; Pilmore et al., 1999) whilst M ϕ phenotype may also change over time as the disease process progresses due to the effects of various different stimuli such as hypoxia, cytokines, apoptotic cells etc. The conflicting findings that are present within the published literature may also reflect the kinetics of the disease process with angiogenesis occurring at early time points followed by microvascular rarefaction that becomes increasingly evident in the long term.

There has been limited study of lymphatic vessels in the context of renal transplantation. Although previous studies examining the restoration of the lymphatic drainage of autotransplanted limbs in a rat model indicated that lymphatic flow is restored but may follow a

more superficial route (Smaropoulos et al., 2005). In fact interest in lymphatics is relatively new and there is debate as to whether the process of lymphangiogenesis in the transplanted organ is inherently good or bad. In the murine model we observed that lymphatic vessels contained M ϕ which had ingested apoptotic cells in addition to T and B lymphocytes. Although this observation certainly supports the theory that these lymphatic vessels are an important exit conduit for the inflammatory infiltrate, it does not indicate that the lymphatic system is completely reconnected. If the development of new lymphatic vessels is key to the clearance of the inflammatory infiltrate from the kidney then this may be beneficial or detrimental. For example, the clearance of injurious cells such as activated M ϕ and cytotoxic T cells is likely to be beneficial and contribute to the resolution of the inflammatory process. In contrast, the facilitation of the trafficking of APCs such as dendritic cells to the lymph nodes may well stimulate the adaptive immune system to continue the alloimmune response against the graft.

We often observed nodular infiltrates of B cells and T cells in the proximity of new lymphatics. Kerjaschki et al reported the presence of nodular infiltrates in a subset of acute rejection tissue with the infiltrates containing B cells, T cells and CCR7⁺ cells whilst the lymphatic vessels expressed CCL21 which is the ligand for CCR7

(Kerjaschki et al., 2004). These nodular infiltrates resemble tertiary lymphatic structures that perpetuate immune responses.

The presence of the B cell is of much interest as recent work suggests that B cells may play an important role in the process of lymphangiogenesis. B cells are present in the tissue of renal grafts undergoing both acute and chronic rejection and are not evident in normal renal tissue (Martins et al., 2007). Angeli et al. noted that B cell expression of VEGF-A is central to the lymphangiogenesis associated with expansion of lymph nodes (Angeli et al., 2006). Cursiefen et al. also noted a key role for VEGF-A in the haemangiogenesis and lymphangiogenesis present in the inflamed rodent eye although the M ϕ was the key cell in this situation (Cursiefen et al., 2002). In the context of acute renal allograft rejection it is interesting to speculate that VEGF-A expression by B cells may be partly responsible for M ϕ recruitment and subsequent VEGF-C expression which stimulates lymphangiogenesis via VEGFR3.

7.2 Future work

Although these studies have provided significant insights into the interaction that may occur between M ϕ and endothelial cells, there are still many questions that remain unanswered and much further work is required before a complete picture emerges.

Human Tissue Studies

The study of the human renal tissue available to us provided pathological information about M ϕ infiltration and the peritubular and lymphatic vessels in CAN. It would therefore be informative to study the microvasculature (PTC number and endothelial proliferation), M ϕ and lymphocyte infiltration and VEGF-A expression of serial biopsies from patients undergoing renal transplantation such that the kinetics of these processes may be determined. In addition, the study and quantification of lymphatic vessels, VEGF-C expression etc in these biopsies would also shed light upon the kinetics of the development of interstitial lymphatic vessels following transplantation. Also, it would be informative to study these same parameters in biopsies from a group of well functioning renal allografts that have been in situ for a comparable length of time as the kidneys affected by CAN. This would indicate whether changes in the number of PTC and lymphatic vessels are found in all transplanted kidneys in the absence of CAN and simply reflect changes that occur over time in the absence of overt pathological disease.

In CAN tissue, unlike control tissue, interstitial M ϕ express iNOS and it would be interesting to undertake triple immunostaining for M ϕ , iNOS and VEGF-C to determine if the same population of Mf may be responsible for both VEC death and LEC proliferation. Since heterogeneous populations of M ϕ exist within tissues, it may be the case that there are phenotypically distinct groups of M ϕ that are responsible for the PTC and lymphatic changes that occur during the development of CAN. Alternatively, the combination of double labelling for M ϕ and VEGF-C and iNOS and VEGF-C double labelling would also provide similar information. For example, if VEGF-C consistently localised to M ϕ but never co-localised with iNOS then this would indicate that the presence of distinct M ϕ populations.

It would also be important to examine the correlation of the changes observed in the potential future experiments outlined with transplant function, structure and outcome i.e. eGFR, proteinuria, scarring and tubular atrophy.

Murine Studies

The work presented in this thesis implicates M ϕ -derived NO in the deleterious PTC rarefaction evident in the acute rejection of a murine renal allograft but the data is not definitive. Further work should include examining the specific effect of iNOS blockade upon acute rejection and PTC attrition in the murine transplant model with the

prediction that microvascular damage would be ameliorated by iNOS blockade. This could be achieved by administering the iNOS-specific inhibitor L-Nil (control D-Nil) in the drinking water (Kipari et al., 2006). An alternative strategy to dissect the role in iNOS would be to transplant kidneys from an iNOS wild-type mouse to an iNOS knockout mouse but this approach is unfortunately complicated by the murine strain requirements of the transplant model (iNOS knockout mice of the FVB/N strain would be required).

Although the *in vitro* studies did not suggest a role for TNF α or FasL, the *in vivo* environment is significantly more complicated and it would be interesting to attempt to block the action of TNF α *in vivo* using function blocking antibodies or soluble receptors. It may be the case that TNF α blockade may modulate the phenotype of infiltrating M ϕ in this model with secondary effects upon the generation of other cytotoxic mediators such as NO.

In the transplant model used in these studies, the acute rejection process does not adversely affect renal function as the normal left kidney is left *in situ*. The future development of a 'functional transplant model' (transplant dependent model) would be of interest. In this case, the recipient would undergo a 2nd nephrectomy at day 5 following the transplant procedure. This model would allow the effect of various interventions upon renal function to be determined.

In the murine model changes occurring during acute rejection have been studied whilst the human tissue studies examined the changes occurring during end stage CAN. It would thus be informative to establish a murine model of chronic rejection such that the role of M ϕ and various M ϕ -derived mediators could be studied in the context of chronic rejection. This would facilitate careful time course experiments to examine the kinetics of the inflammatory and pathological changes and also allow attempts to ameliorate the chronic changes by performing interventional studies such as the administration of VEGF-A, blockade of iNOS or the administration of modified 'anti-inflammatory' M ϕ .

Further work is required to determine the exact mechanisms and function of the lymphangiogenesis described in the murine transplant model. First, however, it will be important to undertake further studies with additional lymphatic markers such as VEGFR-3, prox-1 etc to validate the finding. It would be interesting to perform a digital 3D reconstruction after staining multiple sequential tissue sections to determine if the new lymphatic network communicates with the pre-existing lymphatic system.

At the current time, it is unclear when the lymphatic system of the transplanted kidney becomes 'reconnected' and therefore it would be of interest to undertake protein and cell tracking experiments in

transplants at various time points post-transplantation. For example, fluorescent low molecular weight dextran, carbon particles or fluorescent T cells could be injected into the renal parenchyma with subsequent harvesting of the regional lymph nodes in order to look for evidence of trafficking of these particles or cells by microscopy or flow cytometry of enzymatically dissociated lymph nodes. These studies would shed light upon the time course of restoration of lymphatic drainage following the severance of the lymphatic vessels at transplantation.

It was not possible to assess the proliferation of either vascular or lymphatic endothelial cells using the proliferation marker MIB-1 and further work should employ the administration of Brdu prior to culling the animal. The role of VEGF-C in the lymphangiogenic process needs to be explored further. Immunostaining for VEGF-C expression should be undertaken with double labelling studies with F4/80 being performed to determine whether the VEGF-C⁺ cells are M ϕ . In order to investigate the contribution of lymphangiogenesis to the immunological rejection of the allograft the effect of inhibiting the action of VEGF-C could be undertaken. Possible strategies may employ the administration of functional blocking anti-VEGFR-3 antibody with readouts including markers of lymphangiogenesis as well as the severity of the rejection process.

The involvement of B cells in both rejection and lymphangiogenesis merits further scrutiny and it would be interesting to determine the effect of B cell depletion upon these processes. At the current time, however, an anti-murine B cell antibody is not commercially available.

In vitro work

The murine co-culture studies were undertaken using a cardiac microvascular endothelial cell line as a renal endothelial cell line was not available. Although Mφ often use mediators such as NO to kill various cell types including tubular cells, mesangial cells and tumour cells, it would be informative to perform similar co-cultures with endothelial cells derived from a kidney. Furthermore, interstitial endothelial cells rather than glomerular endothelial cells would be best as there are differences between these 2 types of intrarenal endothelial cells.

It would also be interesting to undertake a series of co-culture experiments involving human monocyte-derived Mφ and human renal endothelial cells as this would indicate the nature of the death effector in a human setting. Previous work has indicated that human monocyte-derived Mφ employ NO, TNFα and FasL in the induction of death of vascular smooth muscle cells (Boyle et al., 2003) and it is

possible that multiple mediators may play a role in the induction of apoptosis of human renal endothelial cells.

My preliminary studies suggesting that endothelial cells in culture consist of a mixture of vascular and lymphatic endothelial cells require additional work to both confirm and extend the findings. If the findings are corroborated in extended studies then they have important implications for the interpretation of studies using vascular endothelial cells and further experiments in this area of endothelial cell research. It will be feasible to isolate pure populations of lymphatic endothelial cells for direct study and comparison with vascular endothelial cells (isolated from the same original mixed population). Further and repeated phenotyping of these cells using additional LEC markers such as VEGFR3, LYVE-1 and prox-1 would be required. Indeed, microarray analysis of the isolated lymphatic and vascular endothelial cells would be interesting to perform as they should exhibit different gene signatures. Ultimately an attempt to perform co-culture studies between M ϕ and isolated lymphatic endothelial cells could be carried out as previously described using the MCEC-1 cells. Also, it would be possible to examine the effect of M ϕ activated with various stimuli upon a '50/50' mixed population of lymphatic and vascular endothelial cells that have been differently labelled with fluorescent dyes so that the fate of the lymphatic and

vascular endothelial cells in the same co-culture can be separately determined.

The in vitro work undertaken in this thesis has utilised 2 dimensional cell culture systems. It would be of great interest to develop studies in a 3D cell culture system as this would facilitate study of the effect of M ϕ upon angiogenesis and the 3D structure of lymphatic vessels and vascular capillaries.

7.3 Clinical relevance and treatment options

We need to first understand the mechanisms of the rejection process in order to consider realistic targets for treatment. Future work will aim to build a comprehensive understanding of the interaction between M ϕ and vascular and lymphatic endothelial cells in the context of renal allograft rejection. Only then can possible treatment targets be sensibly considered but future potential translational strategies may include:

- Inhibition of M ϕ recruitment to the transplanted kidney through the inhibition of key chemokines such as MCP-1.
- Administration of M ϕ that have been modified ex vivo (e.g. treated with cytokines to adopt an alternative activation profile) so that they adopt a reparative or anti-inflammatory phenotype.

- Administration of cytokines (e.g. IL-4, IL-10) systemically with the aim of reprogramming the phenotype of M ϕ within the allograft to a reparative or anti-inflammatory phenotype.
- Modulation of key intracellular signalling pathways such as the NF κ B pathway that is a central regulator of pro-inflammatory gene expression. This may result in increased production of anti-inflammatory cytokines such as IL-10.
- Blockade of key pro-inflammatory cytokines such as TNF α , iNOS etc.
- Administration of mitogens or survival factors such as VEGF-A to support the integrity of renal vascular endothelial cells. The additional effects of mediators such as VEGF-A will need to be carefully considered.
- Modulation of B cell numbers using the B cell depleting antibody Rituximab.

Since both acute rejection and CAN are the result of multiple pathophysiological causes of injury it is likely that there will be no single 'magic bullet' but that several or combined therapies will be needed. Furthermore, such novel therapies will need to be incorporated into the current immunosuppressant drug regimens in use and ideally tailored to the requirements of the individual patient.

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APPENDIX 1

Adair, A., Mitchell, D. R., Kipari, T., Qi, F., Bellamy, C. O., Robertson, F., Hughes, J., and Marson, L. P. (2007). Peritubular capillary rarefaction and lymphangiogenesis in chronic allograft failure. *Transplantation* **83**, 1542-1550.

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